**Universidade de Aveiro** Departamento de Biologia
**2009**

**Catarina Pires Ribeiro Ramos Marques**

# Estudo do Efeito de Pesticidas em Organismos Não-Alvo

# Study of Pesticide Effects on Non-Target Organisms

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## Study of Pesticide Effects on Non-Target Organisms

dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Prof. Doutor Fernando Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação científica da Doutora Ruth Maria de Oliveira Pereira, investigadora auxiliar do CESAM, Universidade de Aveiro.

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Ao meu pai e à minha mãe.

**o júri**

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**palavras-chave**

pesticidas, nutrientes, poluição difusa, cultura intensiva de arroz e milho, minhocas, microalgas verdes, espécies de dafnídeos autóctone e padrão, zona húmida protegida, testes laboratoriais, bioensaios com amostras naturais, bioensaios *in situ*

**resumo**

A utilização insustentável de pesticidas, especialmente em zonas com elevado valor ecológico constitui uma ameaça à integridade dos ecossistemas. Sendo um problema à escala mundial, e também no contexto nacional, o presente trabalho pretende ser um contributo para a avaliação dos efeitos de pesticidas em organismos não alvo terrestres e, principalmente, aquáticos, em contextos de progressiva relevância ecológica. Neste sentido, o estudo foi direccionado para áreas (A1 e A2) integradas numa zona agrícola extensa em Portugal, utilizada para a produção de milho e, principalmente, de arroz (Baixo Mondego), a qual sustenta uma elevada biodiversidade. O estudo teve início na área A1, onde a monitorização físico-química e os ensaios com amostras naturais (ensaios WET - *whole effluent tests*) provenientes desta área evidenciaram que, apesar da ausência de pesticidas, as amostras de água colhidas no canal que atravessava os arrozais foram as mais nocivas para o crescimento de *Pseudokirchneriella subcapitata* e *Chlorella vulgaris*. Uma vez que outras fontes de contaminação (produção de gado) actuavam em A1, o estudo prosseguiu apenas na área A2. Assim, em A2, começou-se por determinar a toxicidade individual e da mistura de dois herbicidas formulados aplicados nos campos de arroz (Viper®) e milho (Mikado®) em condições laboratoriais. Viper® foi o herbicida mais tóxico, tanto para o crescimento de *P. subcapitata* e *C. vulgaris*, como para a sobrevivência, reprodução e crescimento de *Daphnia longispina* e *Daphnia magna*. Adicionalmente, estimou-se que a mistura Viper®/Mikado® induz efeitos antagonistas no crescimento de *P. subcapitata* e efeitos sinérgicos no crescimento de *C. vulgaris* e na sobrevivência dos dafnídeos. A avaliação da toxicidade destes herbicidas formulados e seus ingredientes activos no comportamento de minhocas terrestres (*Eisenia andrei*), usando solos naturais, demonstrou que Viper® e penoxsulam causaram uma % de evitamento superior nos organismos expostos. Contudo, o risco para *E. andrei* será à partida reduzido se as taxas de aplicação dos herbicidas forem respeitadas. Ensaios WET foram novamente usados para testar amostras naturais da área A2. Verificou-se que a qualidade do sistema aquático e do arrozal diminuiu durante a estação agrícola, em paralelo com a presença de nutrientes e pesticidas. O crescimento algal foi inibido, apesar dos parâmetros de história de vida dos dafnídeos terem sido estimulados. O resultado desta avaliação subestimou, em certos casos, os impactos reais causados pela aplicação de pesticidas. A avaliação *in situ* simultânea à aplicação de herbicidas nos arrozais demonstrou que os efeitos registados foram de facto restritos aos pulsos de herbicidas. A inibição das taxas de alimentação de *D. longispina* e *D. magna* forneceram um sinal precoce de alterações no sistema, seguido pela diminuição da sua sobrevivência e do crescimento de *P. subcapitata*. Em suma, as diferentes fases da avaliação efectuada confirmaram a existência de condições desfavoráveis devido às práticas agrícolas, reforçando a necessidade de se conjugar ensaios laboratoriais com avaliações *in situ* de maior relevância ecológica, para reduzir o grau de incerteza aliado à determinação dos riscos.

**keywords**

Pesticides, nutrients, diffuse pollution, intensive rice and corn cropping, earthworms, green microalgae, autochthonous and standard daphnids, protected wetland, laboratorial tests, whole effluent toxicty tests, *in situ* bioassays

**abstract**

The unsustainable use of pesticides, especially in areas with strong ecological value, still threatens the integrity of ecosystems. Being a worldwide problem, also with impact at the national level, the present work pretends to be a contribution for the evaluation of pesticide effects on terrestrial and, particularly, freshwater non-target organisms, following a stepwise approach with increasing ecological relevance. As a way to increase the environmental relevance of the work, some tasks targeted sub-areas (A1 and A2) of an extensive Portuguese agricultural area used for corn and, especially, rice production (Lower Mondego river Valley), which sustain a high biodiversity. The study begun in A1 sub-area. The physico-chemical scrutiny and whole effluent toxicity (WET) assays with the algae *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* showed that, in spite of the absence of pesticides in natural samples, water samples from the canal crossing the rice fields in A1 were the most harmful for the growth of both algae species. Once A1 was constrained by other contamination sources (upstream husbandry areas), it was dismissed from the subsequent work, which was directed to A2. The study in A2 started with the evaluation of the single and mixture toxicity of two formulated herbicides applied in rice (Viper®) and corn (Mikado®) fields, under standard conditions. Viper® was the most toxic herbicide, both for *P. subcapitata* and *C. vulgaris* growth and for the immobilisation, reproduction and growth of *Daphnia longispina* and *Daphnia magna*. The mixture Viper®/Mikado®, under realistic environmental levels, is expected to cause antagonistic effects on *P. subcapitata* growth and synergistic effects on *C. vulgaris* growth, as well as on the immobilisation of daphnids. Furthermore, the toxicity screening of these formulated herbicides and their active ingredients on the behaviour of a terrestrial earthworm (*Eisenia andrei*) using natural soils showed that Viper® and penoxsulam induced higher % of avoidance in exposed organisms. However, the risk for *E. andrei* will be apparently low, if the application rates of herbicides are respected. The toxicity screening of natural samples from A2 (WET assays with water and sediment and soil elutriates) indicated that the aquatic/paddy system quality declined during the cropping season, due to the enhanced input of nutrients and pesticides. This led to inhibitory effects on microalgae growth, while the life-history traits of daphnids were stimulated. This semi-field evaluation was somehow overprotective relatively to the real impacts triggered by intermittent pesticide pulses. Indeed, the *in situ* assessment performed simultaneously to the application of herbicides in rice fields proved that the strongest effects were fairly restricted to the pulses of herbicides. Consistently, the *in situ* decline of *D. longispina* and *D. magna* feeding rates gave an early sign of stress, followed by the decrease on their survival and on *P. subcapitata* growth. Overall, through the evaluating tiers it was possible to confirm the existence of hazardous conditions associated with the farming practices, hence reinforcing the need for conjugate laboratory with *in situ* evaluations, presenting higher ecological relevance, in order to reduce the uncertainty level of risk determination linked to pesticide use.

*Para andar, basta colocar um pé depois do outro.*
*Um pé depois do outro.*
*Não é complicado. Não é difícil.*
*Dá para ter em mente pequenas metas:*
*primeiro só a esquina.*

Adriana Lisboa

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# Chapter I

General introduction

# General introduction

## 1.1 Agrochemicals: environmental exposure and contamination

Natural resources are continuously facing profound changes and quality degradation, mainly triggered by anthropogenic activities, enhanced by the fast growth of human population and subsequent exponential consumption rates. Agriculture is of particular significance in this context, since, on one hand, it is for most OECD countries the major user of national land resources (accounts for 40% of total land area) (Candela 2003, OECD 2008) and, in 2005, 62% of the agricultural area was exploited as arable land by the EU-27 members (EC 2007a). On the other hand, agricultural practices performed on arable land represent a threat to the environment integrity, especially because huge amounts of fertilisers and pesticides are usually sprayed during the cropping seasons (Candela 2003). In Portugal, a surplus in fertiliser (≈ 20%, mainly traduced by inorganic nitrogen fertilisers) and pesticide (26%) use was noticed over the period 1990-1992 to 2002-2004, contrary to the overall decreasing pattern denoted for OECD countries (OECD 2008). The abusive and unsustainable use of agrochemicals will inevitably contaminate environmental compartments which the agro-ecosystems depend on or are adjacent to such as air, soil, surface water/sediment, and groundwater. Although all these environmental compartments are intimately linked, the present work will focus its attention on the soil and, especially, on surface water/sediment compartment.

The agricultural soil is a primary recipient of agrochemicals through direct application, accidental spillages or misuse (Connell and Miller 1984, Candela 2003). Thereby, the soil assumes a major role in what concerns the transport (*i.e.*, through pesticide volatilisation to the atmosphere, runoff of agrochemicals into surface waters, leaching and infiltration of agrochemicals into groundwater), retention (sorption/desorption processes) and degradation (chemical – *e.g.*, photolysis – and microbiological transformations) of agrochemicals, hence regulating their input into adjacent compartments (Connell and Miller 1984, Brown et al. 1995, Wolfe 2001). Considering that agricultural areas are often located nearby aquatic systems, as a way to provide efficient irrigation and drainage facilities (van Wijngaarden et al. 2005), the input of agrochemicals may have as ultimate fate the water bodies. The entry routes of contaminants into that compartment may broadly follow point and/or nonpoint (diffuse) sources (Burgoa and Wauchope 1995, Carpenter et al. 1998, Carter 2000, Wolfe 2001, Reichenberger et al. 2007). Agriculture is regarded as one of the main sources of diffuse pollution worldwide (Loague et al. 1998, Abrantes et al. 2006), contributing with inputs of pesticide and fertilisers into adjacent aquatic ecosystems (Fulton et al. 1999, D'Arcy and Frost 2001). Having this in mind, the present

study will highlight the effects that are likely to occur due to nutrient loading and, particularly, due to pesticide contamination.

The surplus of inorganic nitrogen (N) and phosphorus (P) in surface watercourses and groundwater, beyond their natural occurrences, mainly comes from the overuse of fertilisers on agricultural soils, wastes from livestock production sites or residues of dead organisms, excessive application of manures or sludge, and agricultural, domestic and industrial wastes (Carpenter et al. 1998, Ritter and Bergstrom 2001, Abrantes et al. 2006). In turn, the source of pesticides is generally associated with their intentional application on cropland for agricultural purposes, either by terrestrial or aerial dispersion methods (Ritter 2001). As already mentioned, the major diffuse losses of pesticides to the environment occurring during or shortly after their application are mostly constrained by the degradation and dissipation processes underwent in the top soil layer. The active substance and/or pesticide metabolites can then move along soil surface or profile, either in dissolved or particulate sorbed forms, thereby entering water via artificial drainage systems, surface or sub-surface runoff, and leaching to groundwater. Additionally, aerial spray drift and precipitation can also represent important diffuse inputs of pesticides and their residues to surface water bodies (Burgoa and Wauchope 1995, Carter 2000, Ritter 2001, Candela 2003, Kuang et al. 2003, Abrantes et al. 2006, Reichenberger et al. 2007).

All the environmental processes aforementioned, and especially those that sustain the occurrence of agricultural diffuse pollution are primarily dependent on the mobility of agrochemicals, which in turn is influenced by different interlinked factors: i) climatic conditions, *e.g.*, rainfall events, wind, temperature, light intensity; ii) chemical properties of the active substance, such as water solubility, photochemical and biological degradation half-lives ($DT_{50}$) in different environmental compartments and conditions, vapour pressure, soil/water partition ($K_d$), soil organic carbon/water partition ($K_{oc}$) and octanol/water partition ($K_{ow}$) coefficients; iii) soil properties, like organic matter content, water holding capacity, texture, pH, functional diversity and activity of the microbiological community; iv) agricultural conditions, such as characteristics of the target crop, topography, land tillage management, application profile (*i.e.*, pesticide formulation, amount, rate, moment and techniques) (Brown et al. 1995, Russel 1995, Waxman 1998, Carter 2000, Wolfe 2001, Reichenberger et al. 2007).

Consequently, nonpoint inputs often derive from extensive areas of land and are transported overland, making it difficult to quantify effective chemical emissions and exposure levels to which non-target organisms may be subjected (Carpenter et al. 1998). Notwithstanding, in what concerns pesticides, several studies have determined their occurrence in a range of environmental matrices, for instance, soil, water surfaces, sediment and groundwater, though their overall monitoring is still considerably scarce in the EU (*e.g.*, Candela 2003, Golfinopoulos et

al. 2003, Chelme-Ayala et al. 2005, Guest et al. 2006). Despite their environmental quantification, the pesticides used nowadays are increasingly benign, as opposed to the banned persistent ones (*e.g.*, organochlorine, polycylic aromatic hydrocarbons, polychlorinated biphenyls) (Barr and Needham 2002, Candela 2003, Rohr et al. 2006). In fact, current-use or benign pesticides, generally, do not persist in the environment, most of them being decomposed within few weeks upon sunlight and water exposures. Furthermore, they are metabolised and excreted from the organism, preventing their bioaccumulation (Barr and Needham 2002).

Overall, pesticides mainly comprise herbicides, insecticides and fungicides, although there are also rodenticides, nematicides and acaricides (Waxman 1998). Within each class, the benign pesticides are assigned to some characteristic chemical groups, *e.g.*, organophosphates, carbamates, synthetic pyrethroids, triazines, chloroacetanilides, phenoxyacid herbicides, sulfonamides. Such varied structures also enable them to have more selective and different modes of action (Waxman 1998, Barr and Needham 2002, Candela 2003). However, the intensive and widespread use of pesticides guarantees the prolonged exposure of environmental compartments and non-target organisms to pulses of these non-persistent products, which may induce toxic effects that constrain the sustainability of natural populations. This scenario strengthens the need to implement monitoring and assessment programs, providing a relevant contribution for regulators and managers towards the definition and adoption of management practices to mitigate environmental contamination (Fulton et al. 1999, Carter 2000, D'Arcy and Frost 2001, Reichenberger et al. 2007).

## 1.2 Assessment of pesticide effects

Pesticides are responsible for beneficial effects at different levels: i) ecological – increase land use efficiency by preventing control practices that enhance soil erosion and biodiversity losses (*e.g.*, excessive tillage for pest control); ii) economic – increase of yield rates that leads to the reduction of crop productivity costs through pest control; and iii) social – guarantee food supplies and help controlling human and livestock disease vectors (Russel 1995, Andras et al. 2007, OECD 2008). Nevertheless, since pesticides are biologically active substances, they are able to interact and exert harmful effects on non-target organisms with similar toxicant receptors as the target individuals (van Wijngaarden et al. 2005). Indeed, they are considered as ubiquitous and unique toxic substances or mixtures, designed to kill, repel or harm living organisms (Cox and Surgan 2006, U.S.EPA 2008a). As a way to regulate, prevent, control and reduce unwanted effects of pesticides on human and environmental health, several European Directives have been implemented.

The first regulations addressed the protection of public health with the establishment of Council Directive 80/778/EEC amended by Council Directive 98/83/EC[1] (EC 1998; further amended by Regulation (EC) no. 1882/2003), which defines the quality of water intended for human consumption. In 1991, the Council Directive 91/414/EEC (EEC 1991; meanwhile amended by several law documents) concerning the placing of plant protection products on the market, brought up the awareness about the protection needed against potential environmental exposure and consequent detrimental effects, thereby requiring the assessment of the impact of chemicals on non-target species from different environmental compartments (air, water, sediment, soil). Subsequently, guidance documents were created, namely for conducting terrestrial (EC 2002a) and aquatic (EC 2002b) toxicological assessments in support to the Council Directive 91/414/EEC. Later on, it was developed the EC Technical Guidance Document (TDG; EC 2003) to provide methodologies foreseeing the risk assessment of new notified and existing substances, and substances of concern present in a biocidal product, in support to the Commission Directive 93/67/EEC, Commission Regulation (EC) no. 1488/94 and Council Directive 98/8/EC, respectively. Recently, the REACH Directive (EC 2006) regulates the registration, evaluation, authorisation and restriction of chemicals, hence regarding further implementation of risk assessment processes for existing substances. Meanwhile, the Water Framework Directive[2] (WFD; EC 2000; later on amended by Decision no. 2455/2001/EC and Council Directive 2008/32/EC) was established and a framework concerning the protection of soil (CEC 2006) was recently proposed, though it was not yet approved. In turn, the WFD was supplemented with the Decision no. 2455/2001/EC (EC 2001) establishing the list of priority substances, among which some pesticides were discriminated. The fundamentals of the WFD encompass the protection of aquatic ecosystems and promotion of the sustainable use of water resources. Therefore, WFD requires the monitoring of surface water quality status from each river basin, in order to attain "good" chemical and ecological status as protective goals of the receiving environment.

The assessment or estimation of risks of contamination (*e.g.*, by pesticide use) for the ecosystems/environment may pursue an integrative approach designated as Ecological or Environmental Risk Assessment (ERA), which generally involves collecting, organising and analysing environmental data (U.S.EPA 1998, Jensen and Mesman 2006). There are typically two major types of ERA. It can follow a prospective framework, like it is described in the European TGD (EC 2003), which is linked to the authorisation and handling of chemicals, such as pesticides, and it is ideally undertaken before their environmental release. Or it can be a retrospective risk

[1] Transposition into national law: Law no. 243/2001 (D.R. nº 206, I-Série-A).
[2] Transposition into national law: Law no. 58/2005 (D.R. nº 249, I-Série-A) further corrected by Declaration no. 11-A/2006 (D.R. n.º 39, I-Série-A) and amended by Law no. 77/2006 of 30.03.06 (D.R.nº 64, I-Série-A).

assessment directed to contaminated sites and intended to evaluate or estimate changes occurring in the ecological receptors and overall ecosystem, due to past or ongoing exposures to contaminants (*e.g.*, U.S.EPA 1998, Jensen and Mesman 2006).

Overall, an ERA is often developed in phases or tiers in which may be applied deterministic (*i.e.*, uses fixed values to estimate toxicity, exposure and risks) or probabilistic (*i.e.*, uses models and/or probabilistic distributions to estimate exposure and/or effects and risks) methods. As going through tiers it will increase the effort, time and costs involved, as well as it will refine the study, hence reducing the associated uncertainty (Jensen and Mesman 2006, Maltby 2006).

The scheme or paradigm of an ERA may vary significantly depending on the framework considered. Notwithstanding, two major elements are typically analysed either within the same tier [as in the framework edited by Jensen and Mesman (2006)] or in different tiers [as in the U.S.EPA (1998) and TGD (EC 2003) approaches], which are exposure and effects. Exposure assessment is based on representative measured or experimental data and/or model calculations that provide information about the transport, fate, behaviour and bioaccumulation of contaminants in the environment. Effects assessment allows the identification and quantification of dose (concentration) – response (effect) relationships, through the use of short-term or screening toxicity tests under standard worst case scenarios – lower-tier assessment –, which may proceed to higher-tier evaluations involving ecologically (*e.g.*, field surveys) and environmentally realistic studies (*e.g.*, *in situ* assays). Afterwards, the integration of exposure and effect assessment information will allow estimating, describing and/or characterising the nature and magnitude of risks (Maltby 2006).

In general, the current procedures to evaluate the environmental effects of pesticides are basically disposed on European guidance documents (EC 2002a, 2002b), which are in turn based on different standard ecotoxicological tools. The endpoints that are often surveyed range from lower to more complex biologic organisational levels, including sub-individual (*e.g.*, genetic, biochemical, histopathological, morphological and physiological alterations), individual (*e.g.*, survival, reproduction, growth, behaviour – like feeding inhibition and avoidance), population, community and ecosystem levels. Yet, the sensitivity of the endpoints is inversely related to their ecological relevance (Newman 2001). Usually, the effect assessment starts with lower-tier tools complying single-species toxicity tests run under standard conditions, like the acute, chronic and/or sublethal toxicity tests. Although these evaluations are required for pesticide authorisation and handling purposes (EEC 1991), the available data in open literature barely relies on its acute ecotoxicity. Nevertheless, the acute lethal concentrations of pesticides are usually far above the determined environmental concentrations, which are indeed sometimes below the thresholds for

major sub-lethal effects (Relyea and Hoverman 2006). Even though, the assessment of sub-lethal effects (*e.g.*, behaviour, growth, reproduction) under short- or long-term/chronic exposures strengthens the gap between laboratorial and field exposures.

The standard ecotoxicological tests provide valuable and easily interpretable data, namely for the derivation of toxicity benchmark values, useful to estimate risks and establish allowable levels of contamination (Fleeger et al. 2003). However, they are able to introduce some uncertainty in the risk assessment process, because of their simplistic exposure conditions and reduced ecological relevance (van Wijngaarden et al. 2005, Jensen and Mesman 2006). For instance, chemicals seldom occur alone in the environment. During their spraying, a combination of products may end up in the terrestrial and aquatic systems, which concentrations seemingly non-toxic upon individual exposures can induce a combined toxic action on non-target individuals when mixed up. Therefore, the effect of multiple pesticide exposures should also be assessed and quantified (Relyea and Hoverman 2006, Schular and Rand 2008) and recent studies have been focus on binary and multiple mixture effects of pesticides (*e.g.*, Cedergreen and Streibig 2005, Cedergreen et al. 2007). Furthermore, environmental exposures to pesticides are usually ruled by specific application times and frequencies that generate multiple pesticide pulses into a system. Consequently, the use of continuous exposures throughout a standard test prevents a feasible estimation of the real effects under episodic scenarios of contamination. Additionally, the interaction of environmental natural factors (*e.g.*, nutrient loads and dissolved organic matter) with chemical toxicity is also overlooked under laboratorial conditions (Pardos et al. 1998, Fleeger et al. 2003), as well as the environmental rebound or ecosystem resilience is not coherently assessed (Boxall et al. 2002, Relyea and Hoverman 2006).

Thus, in a way to surpass the uncertainty factors associated with a lower-tier assessment, more refined ecotoxicological tools (*e.g.*, micro- or mesocosms studies, *in situ* studies, field surveys or population, community and ecosystem modelling) have been used to attain an accurate prediction of the realistic impacts of pesticide exposures (Ramos et al. 2000, van Wijngaarden et al. 2005, Maltby 2006). In particular, *in situ* bioassays should be included to optimise an ecosystem quality assessment (Boxall et al. 2002), as they remove laboratory-to-field extrapolations, reduce sampling-related artefacts, allow stressor concentrations to fluctuate naturally, and are cost-effective (Tucker and Burton 1999). Recently, *in situ* tests have been successfully developed for a great variety of organisms (*e.g.*, microalgae, cladocerans, macroinvertebrates, fish), covering a wide range of biological responses (*eg.*, survival, feeding behaviour, growth, reproduction) (*e.g.*, Pereira et al. 2000a, Castro et al. 2004, Jergentz et al. 2004, Domingues et al. 2008). However, just a few of them encompassed the use of autochthonous species as test organisms, which give more ecologically relevant responses than

the standard species, especially when site-specific information is to be generated (*e.g.*, Pereira et al. 2000a, Jergentz et al. 2004).

## 1.3 The culture of rice in the Mediterranean region

Rice, along with wheat and corn, is one of the largest produced cereals in the world, and Asian countries are the top producers (Nguyen 2002). In 2001-2003, rice was the most consumed item (FAO 2006), and has been referred as an important food source for more than half the world's population (Nguyen and Ferrero 2004). In the European Union, Portugal is ranked among the four member states (including Italy, Spain and Greece) assuming higher rough rice areas, production and yield (MED-Rice 2003, Cervelli 2004, FAOSTAT Database 2005). According to the national institute of statistics (INE 2007, 2008), the Portuguese rice producing area has been increasing in the last years, and in 2007 it comprised 27000 ha, which is coherent with the enhanced production rates (≈ 158000 t) recorded for the same year.

In general, the rice culture is held once a year, starting with land tillage in mid April untill September – early October, when rice is harvested. Before sowing, the soil is prepared through different practices, for instance, ploughing, harrowing, and land levelling with laser technology. Simultaneously, the fertilisation process is carried on. Then, rice seeds are dispersed either by plane or terrestrial equipment in late April or in the beginning of May, depending on climatic conditions (MED-Rice 2003).

Rice is grown mostly on fine-textured soils presenting low organic matter (2 - 3 %) and high clay contents, which increases their water retention capacity and, in turn, provides the efficient use of water. This resulting fact is especially relevant because that cereal is cultivated under almost permanent flooded conditions (10 - 15 cm water depth), except for short periods in which paddy fields are drained to enable rice rooting and fertiliser and/or pesticide treatments; a few days later (1 - 2 d), the fields are irrigated again. Such flooded conditions are essential for: i) rice growth and development; ii) regulation of air, water and soil temperature; iii) maintenance of soil oxygenation; iv) reduction of pesticide use to control weeds that normally do not survive under flooded conditions; and v) regulation of availability of soluble nutrients. Hence, the overall irrigation system is sustained by a flow-through series of interlinked irrigation/drainage canals/ditches, in which the water flux is controlled by simple dams or floodgates. Most of the irrigation water comes from nearby water courses and, in turn, they are usually the ultimate fate of water drained from paddy fields, as well (Pereira et al. 2000b, MED-Rice 2003, Cervelli 2004).

Rice culture demands for high consumption of agrochemicals, among which herbicides are the ones applied in higher quantities to limit infesting weeds (*e.g.*, *Echinochloa* spp., *Alisma* spp.,

*Cyperus* spp., *Heteranthera* spp.) and algae proliferation. Additionally, fungicides may be applied against *Magnaporthe oryzae* (anamorph *Pyricularia oryza*), and insecticides are dispersed if *Chironomus* spp. or the red swamp crayfish (*Procambarus clarkii*) affect the normal growth of the plant. Pesticides may be aerially or terrestrially applied and their intensive dispersion could be mainly assigned to two moments - during the emergence of the rice plant (2 - 3 leaves) and during a more developed stage of the cereal (Pereira et al. 2000c, MED-Rice 2003).

The pesticide handling and application practices may therefore facilitate the potential exposure of non-target terrestrial and nearby aquatic compartments. Furthermore, the flooded conditions under which the rice crop is grown, together with the poor permeability of the paddy soil enhance the transport (through drainage and/or runoff) of agrochemical residues into adjacent waterways (Miao et al. 2003, Sánchez et al. 2006). Accordingly, several monitoring studies have detected the presence of pesticides in non-target areas nearby paddy fields, with peak concentrations during and immediately after the moment of application (Jiménez et al. 1999, Cerejeira et al. 2003, Silva et al. 2006). Although the water compartment is the one most extensively monitored, the presence of pesticides in the paddy soil (*e.g.*, Ying and Williams 2000) and in the sediments of inlet/outlet waterways (*e.g.*, Padovani et al. 2006) has already been documented. In fact, Inoue et al. (2002) stressed out the relevance of evaluating pesticide accumulation in paddy soil and sediments deriving from rice fields' drainage, as they both constitute a sink of particulate pesticides.

The rice paddy environment presents unique characteristics that require the elaboration of specific and different assessment scenarios from that of common arable crops. Essentially, simultaneous or consecutive exposures of the same ecological receptors from several environmental compartments (*i.e.*, aquatic and terrestrial paddy systems and neighbouring aquatic system) should be considered. The working group MED-Rice (2003) have been joining efforts towards the harmonisation of European scenarios for assessing rice pesticides, considering an adapted and specific conceptual model combined with a tiered testing strategy (Tarazona and Sánchez 2006). Briefly, the proposed conceptual model basically relies on in-crop (considers different exposure routes in modified aquatic and terrestrial systems, *i.e.*, paddy water and the combination of paddy soil and sediment) and off-crop (directed to the aquatic system surrounding the rice fields) assessments. Regarding the off-crop assessment, it should be noticed that in the Europe (*e.g.*, Portugal, Spain, Italy and Greece), rice is frequently produced in areas of high ecological value (Ramos et al. 2000, MED-Rice 2003, Miao et al. 2003, Cervelli 2004, Padovani et al. 2006, Tarazona and Sánchez 2006), therefore requiring a proper evaluation of pesticide impact, aiming the overall conservation of biodiversity (Tarazona and Sánchez 2006).

### 1.3.1 Lower Mondego Valley - an agricultural area of intensive rice cropping in Portugal

The rice producing areas in Portugal are integrated in the Mediterranean biogeographical region of Europe, which is characterised by a climate that is warm and dry enough to prevent serious rice diseases and to enhance crop yields. The three most important and extensive rice producing areas in Portugal are located in Sado, Tejo/Sorraia and Mondego river basins (MED-Rice 2003, Silva et al. 2006).

The Lower Mondego river Valley is located in the centre of Portugal (40º2'N, 8º43'W) and corresponds to the final section of Mondego river basin, extending between Coimbra and the west coast, spreading along the estuary until draining into the sea. In the last thirty years the average annual temperature from this region was approximately 15.6 ºC and the average annual rainfall was of ≈ 70.1 mm (IM 2008, INAG 2008).

The extensive valley comprises 15000 ha of agricultural land, which is mainly exploited for rice cropping (60%), although corn and beans are also produced in lesser extent (18%). The hydro-agricultural scheme and the rice cultivation practices are similar to what was above described. The agricultural activities in Lower Mondego Valley pursue an Integrated Crop Production scheme, meaning that alternative pest control methods are used aiming the protection of non-target organisms and crop integrity, whilst the use of reduced economic means is prevailed (U.S.EPA 2008b). However, such an approach does not preclude the occurrence of great quantities of nutrient and pesticide inputs into the Mondego river, which provides important resources for different human activities (Anastácio and Marques 1995, Pardal et al. 2002, Cerejeira et al. 2003), namely recreational events, fishing and irrigation water for agriculture. This represents a particular risk for uncultivated areas, such as swamp and wetlands that occur in the valley. They are characterised as surrogate habitats for typical wetland flora and fauna, which has led to the implementation of goals and strategies for their special protection (ICN-RNPA 2002, EEC 1992). Moreover, the irrigation/drainage ditch network sustaining the activities carried out on the Mondego Valley is indeed considered a valuable biological reservoir (Pardal et al. 2002).

The present study focused on two sub-areas mainly used for the rice cropping, both within the Lower Mondego river Valley. The sub-area A1 was placed in Quinta do Seminário, a farm that comprised 70 ha of monoculture rice fields. Quinta do Seminário is situated in the river Pranto catchment basin, a tributary of river Mondego that converges with it in the estuarine area (Pardal et al. 2002, Castro et al. 2005). Thus, the drainage of paddies is made through the main canal crossing the rice fields of Quinta do Seminário – Vala de Enxugo – into the Pranto river, which in turn will discharge in Mondego estuary. Similarly to other rice field areas, this farm holds natural conditions and supply resources for the maintenance of different species. *Lutra lutra* is one of the mammals taking part of Quinta do Seminário marshes, whereas the most typical

waterfowl species are *Ardea purpura*, *Ardea cinerea*, *Circus aeruginosus*, *Milvus milvus* and *Ciconia ciconia*. *Rana perezi* is one of the common amphibian species found. Among the fish community there are non-indigenous individuals (*e.g.*, *Lepomis gibbosus*, *Gobio gobio*, *Gambusia holbrooki*), migrating fish (*e.g.*, *Liza ramada*) and Iberian endemic species (*e.g.*, *Barbus bocagei*). Relatively to the dominant plant species it can be pointed out the presence of the aquatic plant *Myriophyllum* spp. and wetland species (*e.g.*, *Phragmites australis*, *Scirpus lacustris*, *Typha latifolia*). The rice culture in Quinta do Seminário followed similar practices as the ones already explained in the previous section. The agrochemicals used were fertilisers, algicide and pesticides (mainly herbicides) (*c.f.*, chapter II).

The A2 sub-area comprises more extensive agricultural fields than A1, where corn (≈ 5000 ha) and especially rice (≈ 6200 ha) are intensively produced. It is located near Montemor-o-Velho, where a wetland – Paul do Taipal – stands in the proximity of this area. Paul do Taipal was indeed used for rice culture until the 70s decade. Nevertheless, in 1999 it was classified, by national legislation (Law by Decree no. 384-B/99, 23.09.1999), as a special protection area for birds, and thereafter integrated in the Natura 2000 network (EEC 1979, EEC 1992, ICN 2008) (code no. PTZPE0040). Furthermore, in 2001, it was integrated on the Ramsar List of Wetlands of International Importance (Ramsar site no. 1107). Regarding mammals, 22 species could be confirmed, being often identified signs of *Lutra lutra* presence. The 50 ha of that natural marsh supports a great diversity of bird species, since 122 different species were already identified in Paul do Taipal, among which are emphasised *Ardea purpura*, *Circus aeruginosus*, *Pandion haliaetus*, *Ardeola ralloides*, *Hieraaetus pennatus* and *Circaetus gallicus*. Seven amphibian species were recorded in Paul do Taipal, being highlighted two Iberian endemism (*Discoglossus galganoi* and *Triturus boscai*). Among the 6 species of reptiles, *Lacerta schreiberi* is also an Iberian endemism. Among the fish community, the most common species are represented by non-indigenous individuals (*e.g.*, *Lepomis gibbosus*, *Micropterus salmoides*, *Gobio gobio*, *Gambusia holbrooki*), natural species (*e.g.*, *Cyprinus carpio*, *Carassius carassius*), migrating fish (*e.g., Anguila Anguilla* and *Liza ramada*), Iberian endemic species (*e.g.*, *Barbus bocagei* and *Chondrostoma polylepis*), Portuguese endemic species (*e.g.*, *Rutilus macrolepidotus*) and other (*e.g.*, *Cobitis maroccana*, *Gasterosteus aculeatus*, *Atherina* sp., *Platichthys flesus*). In what concerns the typical flora found in Paul do Taipal it is characterised by the presence of the aquatic weed *Myriophyllum* sp., wetland species (*e.g.*, *Phragmites australis*, *Scirpus lacustris*, *Typha latifolia*) and surrounding trees (*e.g.*, *Pistacia lentiscus*, *Olea europea* sylvestris, *Phillyrea latifolia*, *Salix* sp.) (Anastácio and Amaro 1989, Anastácio and Amaro 1992, ICN – RNPA 2002).

Regarding the application of agrochemicals in the sub-area A2, this may occur either through terrestrial or aerial spraying, depending on the farmers' economic resources and the extension of cultured area. The fertiliser commonly applied in rice fields was the same as in the sub-area A1. Among the applied pesticides, herbicides are the ones mostly used (*c.f.*, chapters V and VI). In what concerns the corn crop, it is developed between May and September, again with herbicides being the most used pesticides. They included Atrazerba FL® (500 g atrazine $L^{-1}$), Buctril® (225 g bromoxynil $L^{-1}$), Mikado® (300 g sulcotrione $L^{-1}$), Laddok® (200 g atrazine $L^{-1}$ + 200 g bentazone $L^{-1}$), Primextra S Gold® (370 g atrazine $L^{-1}$ + 290 g metolachlor $L^{-1}$) and Lasso® (480 g alachlor $L^{-1}$). Whenever insecticides are needed, they are applied in restricted zones. Among them, farmers occasionally apply Force® (0.5% tefluthrin) and Karate+® (2.5% lambda-cyhalothrin) (personal communication of farmers and local agricultural engineers).

### 1.3.2 Herbicides: overall use, contamination, and selection

Herbicides are regularly and intensively used during the crop production season, as a way to control a variety of weeds in different crop types. Since 1990's, in a broad sense, herbicides have been the main plant protection product used in arable land, presenting the highest number of different active substances (Candela 2003). In 2002-03, they represented ca. 35 - 38% of the pesticides used in Europe (ECPA 2003, EC 2007b), the highest quantities being usually applied in cereal crops (*e.g.,* rice and corn) (EC 2007b, OECD 2008). In Portugal, an increase in the use of herbicides has been observed, although they have been the second largest pesticide type used in Portugal. In 2006, ca. 2031 t of active ingredients (a.i.) were sold in our country (Vieira 2007).

Most herbicides, by their nature, generally exhibit lower toxicity to animals than other pesticides, because their intended targets are algae and plants (Bowmer 1987, Fairchild et al. 1999, Brock et al. 2000). However, considering that high concentrations of herbicide are needed for an effective herbicidal action, there is a strong potential for the contamination of soil and watercourses (Sabater and Carrasco 1998, Sánchez et al. 2004). Indeed, this is of particular concern in a rice field agro-ecosystem, where different studies have pointed out the dominant quantification of herbicides. Published pesticide concentrations in surface and groundwater were often above the maximum regulated thresholds, either by European (EC 1998) and national (MA 1998) directives establishing surface water quality (0.5 µg $L^{-1}$ for individual pesticides and 2.5 µg $L^{-1}$ for total pesticide content) and water quality criteria for human consumption (0.1 µg $L^{-1}$ for individual pesticides and 0.5 µg $L^{-1}$ for total pesticide content) (*e.g.*, Santos et al. 2000, Tauler et al. 2001, Batista et al. 2002, Candela 2003, Cerejeira et al. 2003, Castro et al. 2005, Silva et al. 2006). Consequently, it is important to assess the impacts of such pesticides on non-target individuals.

In fact, the toxicity of herbicides is mainly addressed for their respective a.i.s. Notwithstanding, herbicides applied on agricultural fields are complex formulations, thereby containing several adjuvants that increase their efficacy against target plants (Tominack 2000, Cedergreen and Streibig 2005, Cox and Surgan 2006). At the same time, adjuvants *per se* are toxic to certain species and, hence, may mediate and/or increase the toxic effect of the whole formulated herbicide, as already documented by different authors (*e.g.*, Stark and Walthall 2003, Cox and Surgan 2006, Bringolf et al. 2007, Pereira et al. in press). Thus, focusing on the ecotoxicological profile the pesticide's a.i.s may underestimate the actual toxicity of the formulated product. Actually, guidance documents developed under the context of directive 91/414/EEC (EEC 1991) for pesticide registration do not require the ecotoxicological assessment of all formulations, just for those whose toxicity could not be extrapolated from that of the a.i. or that have more than one a.i. (EC 2002a, 2002b). Furthermore, the assessment of chronic toxicity, either for the a.i. or the formulation, is only required if the pesticide is applied more than one time, and if its half-life is superior to 2 days. Yet, the acute and, especially, chronic assessment of formulation effects would bring about more reliable and extensive predictions of potential impacts occurring in the different environmental compartments.

Among the formulated herbicides applied in the main rice producing sub-area (A2) targeted in this study, two formulated products were selected to ascertain their ecotoxicological effects. They were Mikado® and Viper® (hereinafter referred as Mikado and Viper, respectively) that are used on corn and rice crops, respectively. Both are relatively new herbicides in the European market (Meazza et al. 2002, Jabusch and Tjeerdema 2005, Bird et al. 2006), albeit the related available ecotoxicological studies are scarce, as far as we are aware.

*a. Mikado*

Mikado is marketed in Europe by Bayer Crop Science. It is a post-emergence herbicide mostly used in corn crops through terrestrial application, for the selective control of broadleaf weeds and annual grasses (Matringe et al. 2005, ter Halle et al. 2006). Mikado is a systemic herbicide that is mainly absorbed by the foliar via. It is produced as a concentrated suspension containing 300 g a.i. $L^{-1}$, and its recommended rate of application is 1.5 - 2 L $ha^{-1}$. It's a.i. is sulcotrione (fig. I.2), a 2-benzoylcyclohexanodione from the triketone class of compounds, which registration occurred in 1991 (Tomlin 2000). The mode of action of sulcotrione relies on the inhibition of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD). In plants and more precisely in chloroplasts, HPPD is involved in the biosynthesis of prenylquinones (include plastoquinones and α-tocopherol) by means of the catabolism of the aromatic aminoacids

phenylalanine and tyrosine. In particular, plastoquinones are important components of the chloroplastic electron-transfer chain at the photosystem II, being also critical cofactors for phytoene desaturase, which in turn is involved in the biosynthesis of carotenoid pigments.

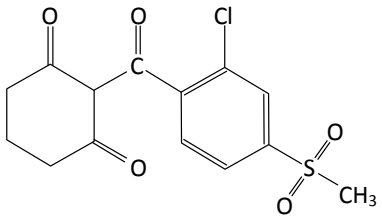


Figure I.2 - Chemical structure of sulcotrione [Source: FOOTPRINT PPDB (2008)].

As such, the inhibition of HPPD in treated plants will lead to the depletion of plastoquinone pools, thus contributing to (i) reduced carotenoid levels externally noticed by the bleaching of plants, (ii) impaired photosynthesis and destabilisation of the respective apparatus as a result of carotenoid loss (essential pigments that protect chlorophyll *a* molecules from strong light intensities). Such impairments will be followed by plant necrosis and death (Mitchell et al. 2001, Meazza et al. 2002, Chaabane et al. 2005, Matringe et al. 2005, Shaner 2003). In mammals, HPPD is also a critical enzyme to tyrosine catabolism, being indeed inhibited by triketone herbicides (Shaner 2003, Matringe et al. 2005). Consequently, it was noticed the accumulation of tyrosine blood levels, which in rats led to serious lesions, while in humans, HPPD inhibitors helped to counteract the symptoms of a genetic disease – tyrosinemia type I – through inhibition of tyrosine catabolism followed by its excretion (Shaner 2003, Matringe et al. 2005). On the other hand, accessed literature did not describe a target site and physiological mode of action for triketone herbicides in invertebrates.

After direct application and/or foliage wash-off, great quantities of sulcotrione may reach the soil. Soil sorption and degradation will rule out sulcotrione mobility and persistence, which in turn is constrained by soil pH, clay and organic matter contents, and the herbicide properties (Chaabane et al. 2008). Table I.3 presents the general physico-chemical characteristics of sulcotrione. Recent works showed that this triketone herbicide presents moderate retention onto soil components (given by $K_d$ and $K_{oc}$ coefficients), being clay content apparently the most conditioning of sulcotrione adsorption (Chaabane et al. 2005, 2008). The dissipation half-lives of sulcotrione in soil varied between 39.9 and 73.5 days (Rouchaud et al. 1998a, 1998b, Chaabane et al. 2008), and according to Rouchaud et al. (1998b) they were longer under basic pH and higher organic matter content.

Table I.3 - Physico-chemical characteristics of sulcotrione and general information about its formulated product. References are indicated on the right side.

| | | |
|---|---|---|
| Chemical name | 2-[2-chloro-(4-methylsulfonyl) benzoyl]-1,3-cyclo-hexanedione | Tomlin 2000 |
| Trade name | Mikado® | http://www.bayercropscience.com |
| Formulation type | Suspension concentrate | http://www.bayercropscience.com |
| Target crop | Corn | http://www.bayercropscience.com |
| Application rate | 1.5 - 2 L $ha^{-1}$ | http://www.bayercropscience.com |
| CAS no. | 99105-77-8 | Tomlin 2000 |
| Chemical formula | $C_{14}H_{13}ClO_5S$ | Tomlin 2000 |
| Molecular weight (g $mol^{-1}$) | 328.77 | Tomlin 2000 |
| Vapor pressure | 5 x $10^{-6}$ Pa at 25ºC | Tomlin 2000 |
| Henry's law constant | 1.00 x $10^{-5}$ Pa $M^3$ $mol^{-1}$ at 25ºC | FOOTPRINT 2008 |
| pKa (dissociation constant) | 3.13 at 23ºC | Tomlin 2000 |
| Water solubility | 165 mg $L^{-1}$ | Rouchaud et al. 1998a, 1998b |
| Log $K_{ow}$ (octanol/water partitioning coefficient) | -1.7 at 20ºC, pH 7 | FOOTPRINT 2008 |
| $K_{oc}$ (soil organic carbon/ water partitioning coefficient) | 74 - 182 | Chaabane et al. 2005, 2008 Cherrier et al. 2005 |
| $K_d$ (soil/water partition coefficient) | 0.25 - 2.52 | Cherrier et al. 2005 |
| Soil degradation $DT_{50}$ | 39.9 - 73.5 days | Rouchaud et al. 1998a, 1998b Chaabane et al. 2008 |
| Aqueous hydrolysis $DT_{50}$ | 100 days at neutral pH, 20ºC | FOOTPRINT 2008 |
| Aqueous photolysis $DT_{50}$ | ND | Chaabane et al. 2007 |
| Aqueous phase DT50 | 9.5 days | FOOTPRINT 2008 |
| Water-Sediment DT50 | 63.9 days | FOOTPRINT 2008 |

Sulcotrione is moderately soluble in water, being hydrolytically stable in darkness, at a pH range 4 - 9 (Chaabane et al. 2007). In fact, Freitas et al. (2004) had already determined the presence of this herbicide in water samples from a lake. It is seldom persistent in the aqueous phase, while in the water - sediment system sulcotrione presents longer half-lives (*c.f.*, table I.3).

In general, two degradation pathways can be found in the soil and water environments, depending on pH, organic matter content and biotic factors (Rouchaud et al. 1998a, Chaabane et al. 2007, 2008). The first one regards the hydrolytic scission between 1,3-cyclohexanedione and the benzoic acid to form CHD (1,3-cyclohexanedione) and CMBA (2-chloro-4-methylsulfonylbenzoic acid), which is the major metabolite. This step could be photoassisted in water environments as confirmed by Chaabane et al. (2007). The other mechanism may lead to the formation of PHD (phenylheptanoic acid derivate), a product that presents 75% herbicidal activity of that shown by sulcotrione. PHD is then further metabolised to CMBA (Rouchaud et al. 1998a, Chaabane et al. 2005, 2008). In plants, sulcotrione may be degraded to CMBA, and in animals, it is rapidly metabolised to 4-hydroxysulcotrione and excreted in the urine (Tomlin 2000, Chaabane et al. 2005). According to WHO, sulcotrione (technical grade) is considered moderately toxic, representing higher toxicity for aquatic plants and freshwater algae than for superior aquatic trophic levels (*c.f.*, table I.4).

Table I.4 - Toxicity data of sulcotrione for different endpoints of some terrestrial and freshwater organisms.

| Organism | Point estimates and toxicity values | | References |
|---|---|---|---|
| **Terrestrial organism:** | | | |
| Worms | 14d-$LC_{50}$ (mg a.i. $kg^{-1}$) | > 1000 | Tomlin 2000 |
| **Aquatic organisms:** | | | |
| Fish: | | | |
| *Oncorhynchus mykiss* | 96h-$LC_{50}$ (mg a.i. $L^{-1}$) | 227 - 390 | Tomlin 2000, Bayer CropScience 2004 |
| mirror carp | " | 240 | Tomlin 2000 |
| *Oncorhynchus mykiss* | 21d-NOEC (mg a.i. $L^{-1}$) | 180 | FOOTPRINT 2008 |
| Crustacea: | | | |
| *Daphnia magna* | 48h-$EC_{50}$ (mg a.i. $L^{-1}$) | 750 | Bayer CropScience 2004 |
| *Daphnia magna* | 21d-NOEC (mg a.i. $L^{-1}$) | 75 | FOOTPRINT 2008 |
| Aquatic Plants: | | | |
| *Lemna gibba* | $EC_{50}$ (mg a.i. $L^{-1}$) | 0.051 | FOOTPRINT 2008 |
| Algae: | | | |
| (unspecified species) | $EC_{50}$ (mg a.i. $L^{-1}$) | 3.5 | AFSSA 2002 |
| *Selenastrum capricornutum* | 96h-$EC_{50}$ (mg a.i. $L^{-1}$) | 1.2 | Tomlin 2000 |
| *Desmodesmus subspicatus* | " | 10 | Bayer CropScience 2004 |

*b. Viper*

Viper (trademark of Dow AgroSciences LLC) is a post-emergence herbicide, being applied to the rice crop via terrestrial or aerial spraying at a rate 2 - 2.5 L $ha^{-1}$, for the selective control of annual grasses, sedges, and broadleaf weeds (Roberts et al. 2003). Viper is a systemic herbicide that is mainly absorbed by leaves, and secondarily by roots. It is an oil dispersion, containing 97.81 % of other ingredients (not specified), including an adjuvant that has methanol (Dow AgroSciences 2002). The a.i. of Viper is penoxsulam [20.4 g a.i. $L^{-1}$] (fig. I.1), a compound that received initial conditional registration in 2004 (U.S.EPA 2004a) and was first approved and launched in EU in 2005 (Bird et al. 2006).

Penoxsulam belongs to the group of triazolopyrimidine sulfonamide (TSA) herbicides that act as acetolactate synthase (ALS) inhibitors. ALS is an enzyme which catalyses the first step in the biosynthesis of branch-chained amino acids (*i.e.*, valine, leucine and isoleucine), which is typical of microorganisms, fungi and plants, being absent in animals. Therefore, it is not expected that penoxsulam could represent a threat to aquatic or terrestrial wildlife. In fact, great efforts have been made to produce herbicides with similar mode of action at plant chloroplasts (Wakabayashi and Böger 2002), as well as the use of TSA herbicides was sincerely greeted, since they were quite efficient and selective in weed control at fairly low application rates, without representing a toxic risk to the environment (Yang et al. 1999).

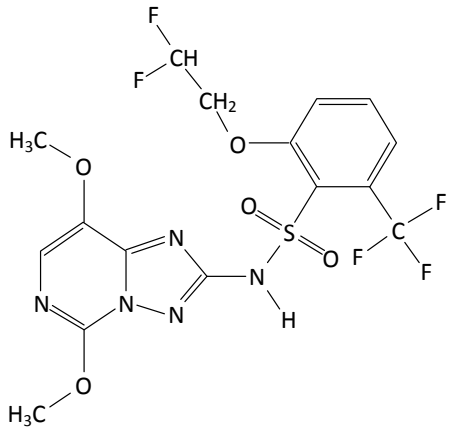


Figure I.1 - Chemical structure of penoxsulam [Source: FOOTPRINT PPDB (2008)].

However, their use induced weed resistance to overall ALS-inhibiting herbicides and alternative ones, besides the negative impacts found on non-target injured plants at residual concentrations (Roberts et al. 2003, Jabusch and Tjeerdema 2006a, Tyler et al. 2007, Jabusch and Tjeerdema 2008).

In plants, the inhibition of ALS activity occurs at the chloroplasts following TSA herbicide treatment, leading to the reduction of aminoacid synthesis. This will induce the rapid cessation of

plant growth, especially at young plant tissues due to the higher amounts of aminoacids needed for protein synthesis (Singh and Shaner 1995, Koschnick et al. 2007). Notwithstanding, the precise mechanisms evolving into growth inhibition and plant death are still under discussion and research, being pointed out several potential secondary effects due to ALS inhibition *e.g.*, accumulation of toxic intermediate compounds (2-ketobutyrate or its transamination product 2-aminobutyrate), disruption of mitosis, disruption of photosynthesis transport, reduction of nutrient transport and assimilation within the plant, changes in mitochondrial electron partitioning thereby affecting respiration rates (Nyström and Blanck 1998, Zhou et al. 2007).

The overall physico-chemical properties of penoxsulam are presented in table I.1. Penoxsulam is expected to be very mobile and non-persistent both in terrestrial and aqueous environments (Roberts et al. 2003). At the soil/sediment compartment, the mobility and persistence of penoxsulam depends on the soil pH, organic matter content, texture, temperature, light intensity and microbial activity. The retention of penoxsulam in soils/sediments is reduced since it has low $K_d$ and $K_{oc}$ partitioning coefficients. However, due to penoxsulam pH-dependent solubility, it could be more strongly bounded to acidic soils/sediments containing high organic matter and clay mineral sorption sites (Roberts et al. 2003, Jabusch and Tjeerdema 2005, 2006a). Penoxsulam soil half-lives vary between 2 - 118 days, depending on the degradation pathway. Anyway, the high mobility of penoxsulam in soil will enhance surface water contamination.

The persistence of penoxsulam in aqueous environment is ruled out by the water pH and temperature, but also by light intensity and microbial activity. It is soluble in water, being hydrolytically stable at the neutral pH range ($DT_{50}$ = 3 – 7 d), although its solubility decreases at lower pHs (Roberts et al. 2003, Jabusch and Tjeerdema 2005, 2006a, 2008). It presents very low vapour pressure and Henry's law constant, thus its volatilisation from water is not likely to occur. The dissipation half-lives for penoxsulam are quite fast ranging between 1.5 – 38 days. Additionally, its low log octanol/water partition coefficient (log $K_{ow}$) also evidences that penoxsulam does not significantly partition into lipids or other organic solvent phases (Jabusch and Tjeerdema 2005, 2008), hence it is not likely accumulated in organisms.

The major degradation pathways of penoxsulam, either in aquatic or terrestrial environments are ruled by photolysis and microbial activity. Photolytic degradation is initially triggered by cleavage of the sulphonamide bridge, whereas the biological pathway proceeds through degradation of the pyrimidine ring and its substituents (Roberts et al. 2003). Penoxsulam can undergo photodegradation in soil at very low rates (U.S.EPA 2004b), although the principal dissipation process is driven by microbial degradation, which is quite rapid under anaerobic conditions typically found in flooded rice field soils ($DT_{50}$ = 2 – 13 d) (Jabusch and Tjeerdema 2006b). On the other hand, its microbial degradation in aqueous phase is slower (U.S.EPA 2004b,

Table I.1 - Physico-chemical characteristics of penoxsulam and general information about its formulated product. References are indicated on the right side.

| | | |
|---|---|---|
| Chemical name | 2-(2,2-Difluoroethoxy)-N-(5,8-dimethoxy-[1,2,4]triazolo[1,5-c] pyrimidin-2-yl)-6-(trifluoromethyl) benzenesulfonamide | U.S.EPA 2004b |
| Trade name | Viper® | http://www.dowagro.com |
| Formulation type | Oil dispersion | http://www.dowagro.com |
| Target crop | Rice | http://www.dowagro.com |
| Application rate | 2 - 2.5 L $ha^{-1}$ | http://www.dowagro.com |
| CAS no. | 219714-96-2 | U.S.EPA 2004b |
| Chemical formula | $C_{16}H_{14}F_5N_5O_5S$ | FOOTPRINT 2008 |
| Molecular weight (g $mol^{-1}$) | 483.4 | Jabusch and Tjeerdema 2008 |
| Vapor pressure | 9.5 x $10^{-14}$ Pa at 25ºC | Jabusch and Tjeerdema 2008 |
| Henry's law constant | 1.66 x $10^{-16}$ atm $M^3$ $mol^{-1}$ at 25ºC | Dow AgroSciences 2006 |
| pKa (dissociation constant) | 5.1 (ambient) | U.S.EPA 2007 |
| Water solubility | 5.7 mg $L^{-1}$ at pH 5<br>410 mg $L^{-1}$ at pH 7<br>1460 mg $L^{-1}$ at pH 9<br>(creates an emulsion) | Roberts et al. 2003 |
| Log $K_{ow}$ (octanol/water partitioning coefficient) | 71.137 at pH 5<br>8 - 0.602 at pH 7<br>9 - 1.418 at pH 9 | U.S.EPA 2007 |
| $K_{oc}$ (soil organic carbon/ water partitioning coefficient) | 104 | Dow AgroSciences 2006 |
| $K_d$ (soil/water partition coefficient) | 0.13 - 1.96 | U.S.EPA 2007 |
| Soil anaerobic $DT_{50}$<br>Soil aerobic $DT_{50}$ | 2 - 13 days<br>34 - 118 days | Jabusch and Tjeerdema 2006b<br>U.S.EPA 2007 |
| Aqueous anaerobic $DT_{50}$<br>Aqueous aerobic $DT_{50}$<br>Aqueous photolysis $DT_{50}$ | 5 - 11 days<br>12 - 38 days<br>1.5 - 14 days | U.S.EPA 2007 |

2007) (*c.f.*, table I.1), being photolysis the predominant route of penoxsulam dissipation in the water system ($DT_{50}$ = 1.5 – 14 d), under favourable light conditions (U.S.EPA 2007). Overall, thirteen products are produced upon penoxsulam transformation. Among them eleven are classified as major metabolites and six are considered of special toxicological concern (BSTCA, 2-amino-TCA, 5-OH-penoxsulam, SFA, 5,8-diOH, sulfonamide). Nevertheless, they are non-persistent and more difficult to detect in the environment than the original compound (U.S.EPA 2004b 2007, Jabusch and Tjeerdema 2006a, 2006b).

According to U.S.EPA (2004b), the application of penoxsulam at proposed maximum levels represents a potential risk to aquatic and terrestrial non-target plants. However, the results of the screening-level risk assessment suggested that penoxsulam would not represent a threat to aquatic or terrestrial animals, what is in straight compliance with WHO (2005) classification for penoxsulam, according to which, penoxsulam is unlike to produce acute hazard under normal use. Actually, the available ecotoxicological data regarding the effects of penoxsulam are fairly scarce and almost limited to its acute toxicity, as it is shown in table I.2.

Table I.2 - Toxicity data of penoxsulam for different endpoints of some terrestrial and freshwater organisms.

| Organism | Point estimates and toxicity values | |
|---|---|---|
| **Terrestrial organism:** | | |
| *Eisenia fetida* | 14d-$LC_{50}$ (mg a.i. $kg^{-1}$) | 1000 |
| **Aquatic organisms:** | | |
| Fish: | | |
| *Oncorhynchus mykiss* | 96h-$LC_{50}$ (mg a.i. $L^{-1}$) | > 102 |
| *Pimephales pomelas* | 21d-NOEC (mg a.i. $L^{-1}$) | 10.2 |
| Crustacea: | | |
| *Daphnia magna* | 48-$EC_{50}$ (mg a.i. $L^{-1}$) | 98.3 |
| Aquatic plants: | | |
| *Lemna gibba* | $EC_{50}$ (mg a.i. $L^{-1}$) | 0.003 |
| Algae: | | |
| *Anabaena flos-aquae* | Acute-$EC_{50}$ (mg a.i. $L^{-1}$) | 0.27 |

Sources: Dow Agrosciences (2006), FOOTPRINT (2008).

## 1.4 Objectives and structure of the thesis

The unsustainable use of pesticides, especially in areas with strong ecological value is one of the utmost threatening factors contributing for the degradation of environmental quality, while the rebound capacity of the environment is getting surpassed. Consequently, biodiversity and

habitat losses are major concerns under an environmental point of view, thus constituting one of the driving forces for the development and implementation of comprehensive ecological risk assessment programs.

Bearing this in mind, together with all aspects focused above, the main goal of the thesis concerned the evaluation of pesticide effects on non-target organisms, belonging to the terrestrial and, especially, to the aquatic compartments. In particular, the present work also intended to assess overall changes in ecosystem quality and integrity, potentially driven by the application of pesticides in areas of intensive agricultural production mainly directed to the culture of rice. The data gathered throughout the study will be a valuable contribution for a future environmental risk assessment of the study area, especially regarding the contamination triggered by rice fields. On the other hand, the derivation of standard ecotoxicological data from formulated products improves the available information that is useful for the registration and notification processes.

First of all, we focused on the A1 sub-area, attempting an initial screening of water quality from watercourses adjacent to the rice fields. However, no further work was done in that area due to the co-occurrence of different contamination sources along the river Pranto (*e.g.*, husbandry areas), which was the origin of irrigation water for the flooding of rice fields. The second part of the study was directed to the sub-area A2, in which a more comprehensive assessment of exposure and effects was pursued. In order to achieve these purposes, different complementary tools were used towards a refined assessment of the effects generated in A2. Tools covering lower tier (laboratorial acute and chronic tests), semi-field (WET tests) and higher tier (field bioassays) levels were used to evaluate impacts under *in situ* conditions.

Thus, following a tiered rationale, this thesis is structured in seven chapters. The first and seventh chapters concern the general introduction and final remarks of the thesis, respectively, while the other five are individual research papers *per se*, published or submitted to peer-reviewed journals. The second chapter regards the first part of the study held in the sub-area A1, while the remaining four chapters (III - VI) were associated with the second study sub-area A2, each one focusing on specific assessment tiers. The chapter objectives, which in turn correspond to the specific objectives of the thesis, are briefly described below.

[Chapter II] Our goal was to evaluate the toxicity of natural samples (*i.e.*, field water and sediment elutriates) collected in a monoculture farm (A1) during the drainage of rice fields, through the combination of physical and chemical analysis and laboratorial WET assays with two freshwater algae species (*Pseudokirchneriella subcapitata* and *Chlorella vulgaris*). This chapter is a contribution for monitoring the quality of water resources surrounding areas of intensive rice

production, under the WFD requirements (EC 2000). In parallel, it provides a prediction of potential hazards in the receiving environment, while integrating interactions occurring in a complex field sample.

[Chapter III] In order to determine the acute and/or sub-lethal effects of Viper and Mikado, as well as their mixture, it was conducted toxicity assays on two primordial freshwater trophic levels: green microalgae (*P. subcapitata* and *C. vulgaris*) and daphnids (*Daphnia longispina* and *Daphnia magna*). The outcome improves herbicide ecotoxicological profiles and enlarges aquatic toxicity databases, which are essential for the development and definition of benchmark values for these products. Such criteria are welcome and needed at the screening-level of an ERA process to support decisions regarding the subsequent steps.

[Chapter IV] We aimed at determining the sub-lethal effects of Viper and Mikado, and their respective a.i.s, on earthworm avoidance behaviour, using natural soils. This chapter will provide improved knowledge about herbicide sub-lethal ecotoxicity on terrestrial ubiquitous organisms and will also contribute to increasing the ecotoxicological data regarding the soil compartment, whose contamination and associated risks have been fairly neglected until recently.

[Chapter V] This chapter's objective was to evaluate the toxicity of natural samples (*i.e.*, field water and sediment elutriates) collected in sites with different impact degrees, located in an agricultural area predominantly exploited for rice culture and that is nearby a protected wetland. Water and sediment/paddy soil quality was compared before and during the cropping season, using the same tools pointed out in Chapter II. However, this assessment was more comprehensive and encompassed the use of two trophic levels - green microalgae (*P. subcapitata* and *C. vulgaris*) and daphnids (*D. longispina* and *D. magna*).

[Chapter VI] Finally, we performed an *in situ* time-scale assessment of the effects generated on caged organisms from two sensitive freshwater trophic levels – microalgae (*P. subcapitata*) and daphnids (*D. longispina* and *D. magna*) – upon the application of herbicides on rice fields by farmers. Survivorship and sublethal (*i.e.*, growth and feeding inhibition) endpoints were monitored along with the regular physical and chemical scrutiny. This higher-tier approach provides the most feasible and real outcome of the impacts of pesticide diffuse pollution on the aquatic system.

The final outcome of this thesis is to generate new ecotoxicological data for the selected herbicides, both for the terrestrial and, especially, for the aquatic compartments. Additionally, the development of a site-specific assessment on a study area mainly exposed to agricultural diffuse contamination sources is expected to provide information about the potential impacts and risks to the ecosystem. This is particularly relevant whenever a protected wetland is located nearby, as long as it regularly sustains a considerable wildlife biodiversity pool, whose protection goals are clearly established in regulatory demands (*e.g.*, EEC 1992, EC 2000). Furthermore, under the Portuguese context, there is a severe lack of knowledge about the effective environmental threats triggered by anthropogenic activities, namely those associated with the use of agrochemicals. Hence, the results herein generated also contribute for the optimisation of eventual management processes to be applied to an area with high economic and ecological value.

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# Chapter II

Are *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* affected by environmental samples from a rice field?

# Are *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* affected by environmental samples from a rice field?

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**Abstract**

Rice fields represent important production rates in Portugal. However, the intensive soil management and the use of agrochemicals may pose a threat to non-target organisms. Hence, the present work regards the toxicity screening of surface waters and sediment elutriates collected during the drainage of fields in the vicinity of a rice paddy (Quinta do Seminário, Soure, Portugal): 1. in River Pranto (RP), the river from which the field irrigation water is canalised; 2. inside the rice paddy, from the main drainage canal – Vala de Enxugo (VE). For that purpose, it was used a combination of physico-chemical analyses and bioassays with two green algae species – *Pseudokirchneriella subcapitata* and *Chlorella vulgaris*. The chemical screening showed an apparent absence of xenobiotics in sediment samples, while no pesticides were found within the chemical contaminants detected in water samples. The nutrient load reflected low levels of organic contamination. Bioassays revealed that *P. subcapitata* was more sensitive to the overall physico-chemical conditions in natural samples than *C. vulgaris*, being its growth inhibited under water samples from both sites. On a whole, water samples, mainly those from the main irrigation/drainage canal of the rice fields (VE), were more deleterious to microalgae than those from RP or any of the elutriates.

**Key-words:** agrochemicals, green algae, growth inhibition, natural samples, rice fields, WET tests.

## 2.1 Introduction

Rice is the second largest produced cereal in the world (UNCTAD 2005), and Portugal is the fourth European country assuming higher production rates (FAOSTAT Database 2005).

Notwithstanding, the contamination of watercourses could be high in areas where rice is cultivated, especially under flooded conditions (Miao et al. 2003). The irrigation system of rice fields enhances the likelihood of the transport of agrochemicals (either fertilisers or pesticides) to the surrounding irrigation/drainage canals and waterways via run-off, direct overspray, aerial spray drift, accidental spills and leaching (Albanis et al. 1998, Cerejeira et al. 1998, Sabater and Carrasco 2001, Cerejeira et al. 2003, Miao et al. 2003, Sánchez et al. 2004, Phyu et al. 2005, van Wijngaardrn et al. 2005, Padovani et al. 2006). Hence, non-target species in the vicinity of this kind of agricultural areas are potentially at risk (Sabater and Carrasco 1998, Geoffroy et al. 2004, Ma 2005, Phyu et al. 2005).

Therefore, it is noteworthy to do every endeavour for regular performance of water quality surveillance in rice culture areas. Indeed, the Water Directive Framework (WDF) (EC 2000) strengthens the need to monitor water quality to assess the impact of human activities on surface waters from each river basin. Within the WDF, targets are described for 'good chemical status' and 'good ecological status' (Whitehouse et al. 2004).

In this context, additionally to the physico-chemical scrutiny, one of the biologically-based approaches applied in hazard assessments of field collected samples on non-target organisms, concerns the use of Whole Effluent Toxicity (WET) testing (U.S.EPA 2002a). WET and similar toxicity tests integrate interactions among complex mixtures of contaminants (Chapman 2000, Wharfe 2004, Wharfe et al. 2004). They measure the total toxic effect, regardless of physical and chemical composition (Chapman 2000), being considered effective tools for predicting instream impacts induced by different kinds of contamination sources (Ausley 2000, de Vlaming et al. 2000, Diamond and Daley 2000, Hutchings et al. 2004). Notwithstanding, similar procedures have already been applied in bioassays with sediment elutriates to evaluate their toxicity (Cheung et al. 1997, Pardos et al. 1998, Wong et al. 1999, Baun et al. 2002, Müller et al. 2002), as sediment acts as a sink of contaminants, which can be exchanged between the sediment and the aqueous phase due to disposal, stormwater runoff and water turbulence (Ankley et al. 1991, Mucha et al. 2003), which is likely to occur during the drainage of rice fields.

Microalgae are frequently used in ecotoxicological studies to evaluate possible negative effects of different kinds of environmental samples (*e.g.*, wastewater, leachates, surface water, soil and sediment elutriates), as well as chemicals and preparations (Geis et al. 2000, Eisentraeger et al. 2003, Geoffroy et al. 2004). The applicability of algae as test organisms is attributed not only to their main functional role as primary producers on trophic chains, being responsible for energy

and nutrient cycling, but also because they are sensitive to a number of pollutants, have high reproductive rates (thereby giving quick indications of the contamination pattern) and are easy to cultivate under laboratory conditions (Nyholm and Källqvist 1989, Campanella et al. 2000, U.S.EPA 2002b, Källqvist and Svenson 2003, Geoffroy et al. 2004).

Hence, the main focus of the present work regards the toxicity screening of surface waters and sediment elutriates collected during the drainage of fields, in two sites from the vicinity of a rice paddy located in the centre of Portugal. For that purpose, it was used a combination of physico-chemical analyses (xenobiotic screening analyses, pH, un-ionised ammonia, nitrate and phosphate) and the comparison of growth responses of two green microalgae species – *Pseudokirchneriella subcapitata* and *Chlorella vulgaris*.

## 2.2 Material & Methods

### 2.2.1 Study area, rice cropping and sampling strategy

Quinta do Seminário (fig. II.1) (with the geographic co-ordinates 40º2'N, 8º43'W) is a farm that comprises 70 ha of rice fields, being situated in the River Pranto catchment basin, a tributary of river Mondego (Castro et al. 2005). It is integrated in a littoral centre region where rice is intensively produced – Lower Mondego river Valley. The rice cropping is supported by an organised system of irrigation/drainage canals around the paddies. The water flux in those canals is controlled by small and simple dams, constructed in strategic points to provide the entrance or discharge of water. The drainage of paddies is made through the main canal – Vala de Enxugo – to the river.

The rice crop in the River Pranto catchment basin and in the whole littoral centre region starts with land tillage usually in late April and grows until September, when rice is harvested. The application of agrochemicals over fields occurs mainly during the end of April up to June, but additional amounts of fertilisers or pesticides are amended along the whole cropping season, depending on the type of culture demands and the rice crop regional conditions. Hence, the fertiliser applied is ammonium sulphate and the algicide is copper sulphate. The mainly applied pesticides, in our study area, were herbicides, being Roundup Ultra® (360 g gliphosate $L^{-1}$; solution), Ronstar G® (2% oxadiazon; granule), Stam Novel Flo 480® (480 g propanil $L^{-1}$; sprayable concentrate), Ordram® (7.5% molinate; granule) and Basagran® (480 g bentazone $L^{-1}$; solution) the ones mostly dispersed.

The collection of environmental samples was performed along with the drainage of fields (in August 2004), thereby, it was not simultaneous to the main pesticide application period. That sampling moment was chosen, once we intended to assess possible risks to the surrounding

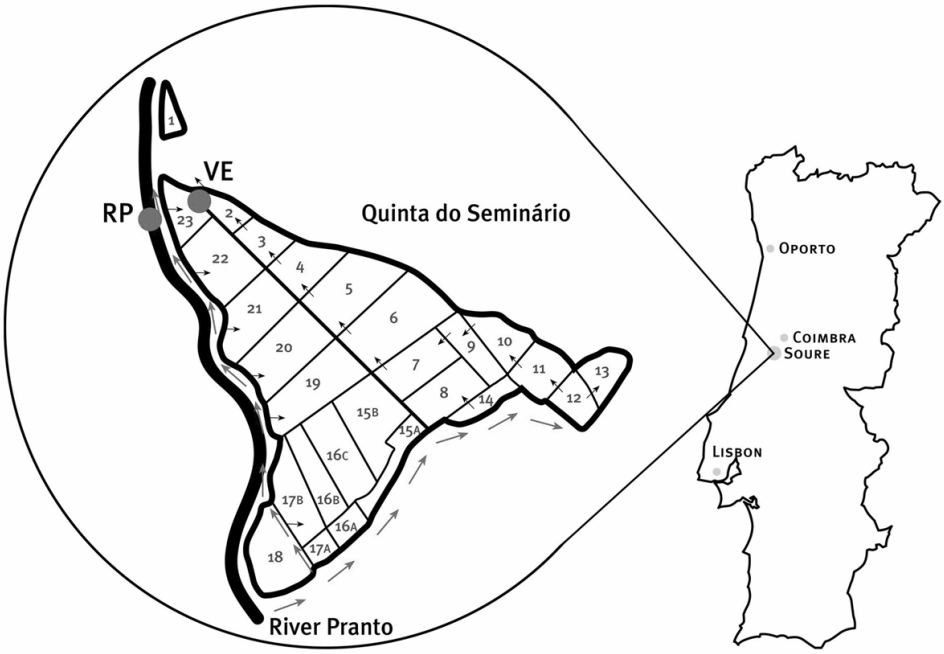


Figure II.1 - Representative scheme of the rice field (Quinta do Seminário, Soure, Portugal). Rice plots are numerated and the arrows indicate the water flow direction of fields' irrigation. RP (River Pranto) and VE (Vala de Enxugo) are the sampling sites.

aquatic system, due to pesticides potentially adsorbed to soil particles, which are transported during the draining process. Two sites were selected in the River Pranto catchment basin: the main drainage canal – Vala de Enxugo (VE) – and in River Pranto (RP), upstream the discharging point of the water coming from the rice paddy (fig. II.1). The selection of the study sites was done considering that VE is potentially more exposed to agrochemicals than RP. Subsurface water samples were collected to 20 L containers. Sediment sampling was conducted according to U.S.EPA guidelines (2001), by using a stainless steal corer with 11 cm diameter and 29.5 cm height. In the lab, water samples were stored in dark at 4ºC, until testing. In turn, sediments were homogenised and sieved in a 2-mm mesh size sieve, transferred to plastic containers already covered with aluminium foil, and stored in the same conditions of the water samples.

### 2.2.2 Preparation of water samples and elutriates

Before testing, water samples (W) from both sites (VE-W and RP-W) were filtered through GF/C filters (U.S.EPA 2002a).

Sediment elutriates were prepared 2 days before bioassay performance. The followed procedures were adapted from Nebeker et al. (1984) and Ankley et al. (1991). In order to discard the nutrient deficiency in the sediments from VE and RP, they were mixed with Woods Hole nutritive culture medium MBL (Stein, 1973), a synthetic medium used for algal culture, in a

volumetric sediment-to-MBL ratio of 1:4 and placed in an orbital shaker for 2h at ≈ 200 rpm. Then, the samples were allowed to settle overnight. The supernatant was siphoned off and centrifuged at 5000 rpm for 15 min., at 4ºC. The obtained elutriates (E; VE-E and RP-E) were filtered in the same conditions as the water samples.

### 2.2.3 Physical and chemical analyses

Nutrient analyses were performed before initiating the test, following the Hach test methods for the determination of nitrate ($NO_3^-$ -N), un-ionised ammonia [$NH_3$-N; the most toxic form for the aquatic organisms (Koukal et al. 2004] and phosphate ($PO_4^{3-}$) in water samples and elutriates. A qualitative chemical screening of water and sediment samples was performed after the acidification of samples. The extraction procedure was carried out according to the method no. 3550B for solid phase extraction (U.S.EPA 1996). The gas chromatography-mass spectrometry (GC-MS) analysis was performed according to the method no. 8270C, included in the same manual (U.S.EPA 1996).

### 2.2.4 Algal cultures and bioassays

Unialgal inoculum cultures of *Chlorella vulgaris* Beijerinck and *Pseudokirchneriella subcapitata* (Korshikov) Hindak were maintained in 250 mL Erlenmeyer flasks with 100 mL of sterilised MBL in an incubator chamber, with controlled temperature (20 ± 2ºC) and photoperiod ($16^L$:$8^D$h), with light provided by cool-white fluorescent lamps.

Bioassays were conducted according to EPA (U.S.EPA 2002a) and OECD (2002) guidelines. The tested dilutions were 12.5, 25, 50, 75 and 100% of water or elutriate, being the dilution water the MBL medium. Simultaneously, the dilutions of 12.5, 25, 50 and 75% of MBL with distilled water were also tested to discard negative effects due to the possible lack of nutrients in field samples. Initial cell densities were approximately $10^4$ cells $mL^{-1}$. The bioassays were performed for three replicates of each treatment plus the control, under constant agitation (≈ 100 rpm in an orbital shaker) and in the same conditions of algal cultures. Light intensity remained between 90.98 and 108.16 µmol $s^{-1}$ $m^{-2}$ (or 4665.64 and 5546.66 lux). *P. subcapitata* and *C. vulgaris* were exposed to water and elutriates from VE and RP during 96h. At the end of the bioassay, the pH was measured to ensure that there were no oscillations and the cell density (counting of cells on a microscope Olympus CKX41 using a Neubauer chamber) was determined as a biomass parameter. The endpoints growth rate (GR; $day^{-1}$) and percentage of growth inhibition (% I) were calculated from cell density measurements.

### 2.2.5 Statistical analysis

One-Way ANOVA followed by a Dunnett´s test for multiple comparisons was employed to ascertain if growth rates determined to each treatment were significantly different from those of the control (Zar 1996). No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were taken from ANOVA significant results, reported for $P < 0.05$. The point estimate $IC_{20}$ (%), corresponding to the concentration that induced 20% of algae growth inhibition during the 96h exposure period was achieved from Probit regression analysis (Finney 1971). A lower effect level was chosen, since it provides a conservative estimation of the potential impacts affecting microalgae populations, without compromising the ecological integrity.

## 2.3 Results

In general, the physico-chemical parameters assumed higher values in elutriate than in water samples (table II.1). The RP-W sample presented higher concentrations of un-ionised ammonia (0.21 mg $L^{-1}$), nitrate (1.90 mg $L^{-1}$) and phosphate (0.30 mg $L^{-1}$), in opposition to VE-W (0.19, 0.20, 0.06, respectively). In elutriate samples great concentrations of un-ionised ammonia and nitrate were detected in VE-E (4.56 mg $L^{-1}$ and 8.00 mg $L^{-1}$, respectively). Overall, the pH values varied between 6.8 and 8.0.

The qualitative chemical screening revealed no detectable xenobiotics among the sediments analysed from VE and RP. However, several chemical contaminants (not pesticides) were detected in water samples from both sites (table II.2).

Table II.1 - Physico-chemical data determined in water samples and elutriates from VE and RP: pH, concentration of ammonia ([$NH_3$-N]), nitrate ([$NO_3^-$-N]) and phosphate ([$PO_4^{3-}$]) (mg $L^{-1}$).

| | Sites | pH | [$NH_3$-N] | [$NO_3^-$-N] | [$PO_4^{3-}$] |
|---|---|---|---|---|---|
| Water | VE | 6.8 | 0.19 | 0.20 | 0.06 |
| | RP | 7.7 | 0.20 | 1.90 | 0.30 |
| Elutriates | VE | 7.9 | 4.56 | 8.00 | 0.01 |
| | RP | 8.0 | 0.21 | 7.40 | 1.42 |

During the algal bioassays, the validity criteria were in accordance with the guidelines of EPA (U.S.EPA, 2000) and OECD (2002). There was no significant reduction of both microalgae growth, when they were exposed to dilutions of MBL with distilled water, meaning that *C. vulgaris* and *P. subcapitata* were not under deficiency of nutrients.

Table II.2 - Qualitative chemical analysis of water samples from VE and RP (ng $L^{-1}$). The method detection limit was 0.20 ng $L^{-1}$.

| Chemical compounds | Samples | |
|---|---|---|
| | VE - W | RP - W |
| Methyl-isopropyl-sulphuret | >0.20 | >0.20 |
| Ethanedioic acid | >0.20 | >0.20 |
| 3-methyl-2-pentanedioic acid | >0.20 | >0.20 |
| Tetrachlorothiophene | <0.20 | >0.20 |
| Benzaldehyde | >0.20 | >0.20 |
| Naphthalene | <0.20 | >0.20 |
| Methylmalic acid | >0.20 | >0.20 |
| 3-Hydroxyvaleric acid | >0.20 | >0.20 |
| Vanillin | >0.20 | >0.20 |
| 1,1'-Biphenyl | >0.20 | >0.20 |
| Phenoxyguaiacyl | >0.20 | >0.20 |
| 7-methyl-6-aminopurin | >0.20 | >0.20 |
| Bi(2-ethylhexyl)phthalate | >0.20 | >0.20 |
| beta-sitosterol | >0.20 | >0.20 |

*VE-W* - water from VE; *RP-W* - water from RP.

The results respecting field samples suggested that the growth rate of *P. subcapitata* was significantly depleted under any dilution of water samples from VE [$F_{(5,12)} = 98.1$; $P < 0.05$] and RP [$F_{(5,12)} = 39.3$; $P < 0.05$], being the LOECs similar and ≤ 12.50% of water (fig. II.2, table II.3). In spite of this, the percentage of water sample needed to inhibit 20% ($IC_{20}$) of *P. subcapitata* growth under VE-W (29.9 %) was lower than the one achieved for RP-W (58.8 %) (table II.3).

Notwithstanding, the growth rate of *C. vulgaris* pursued a different trend from that observed for *P. subcapitata*, as a significant reduced growth caused by VE-W [$F_{(5,12)} = 20.7$; $P < 0.05$] and RP-W [$F_{(5,8)} = 33.3$; $P < 0.05$] was limited to the 100% of each sample (LOEC ≥ 100%), whereas, in lower dilutions, a stimulation growth rate pattern (either significant – only for RP-W – or not significant) could be retrieved from the occurrence of negative % I (fig. II.3). Thereby, *C. vulgaris* exhibited a significant biphasic response, reflecting a low dilution stimulation *vs.* a high dilution inhibition, when exposed to RP-W. Contrary to what was verified in *P. subcapitata*, the $IC_{20}$ of *C. vulgaris* exposed to water samples were impossible to calculate, due to the negative values of the endpoint %I (fig. II.3, table II.3).

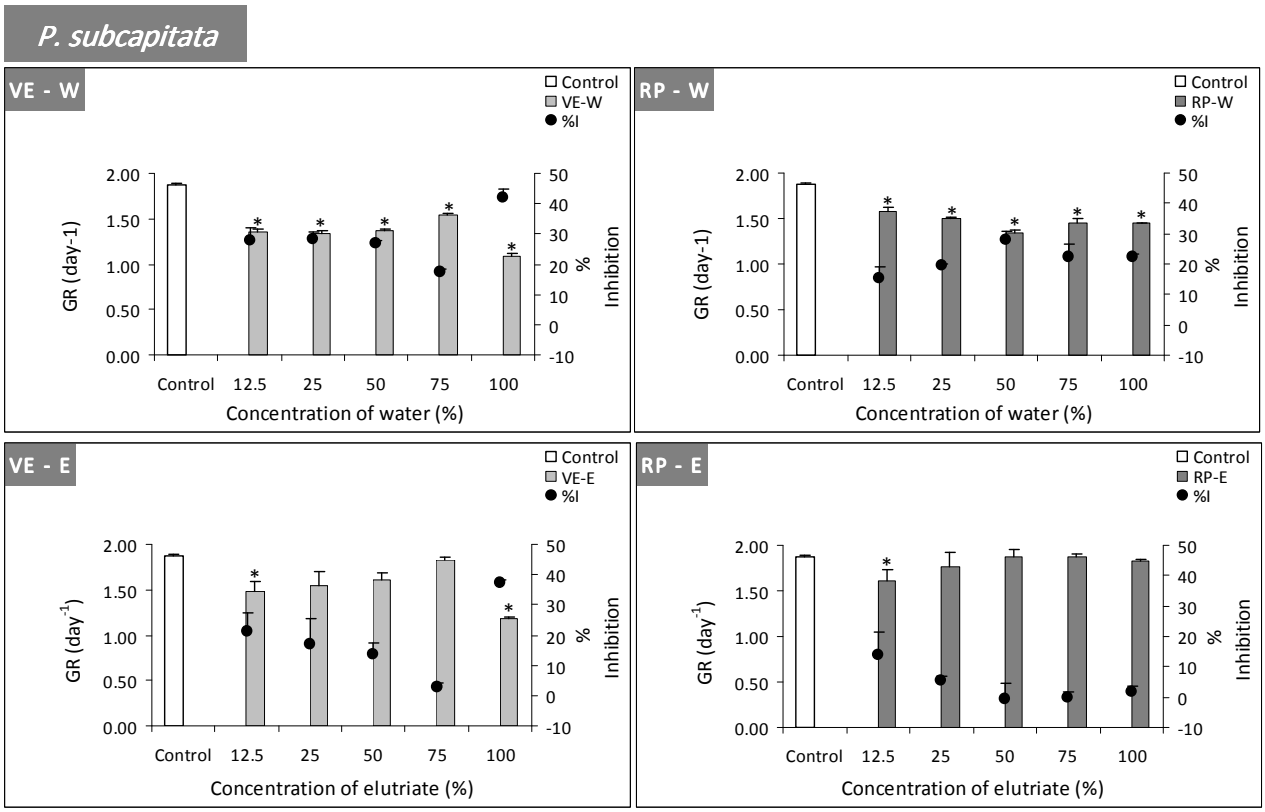


Figure II.2 - Growth rates (*GR*, day$^{-1}$) and % of inhibition (*%I*) for *P. subcapitata* exposed to water samples and elutriates from VE (*VE-W* and *VE-E*, respectively) and RP (*RP-W* and *RP-E*, respectively). Error bars represent standard deviation and the asterisks indicate a value significantly different from the control $P < 0.05$.

Elutriate samples from VE (VE-E) induced a $IC_{20}$ of 73.6% for *P. subcapitata* (table II.3), being the growth rates significantly different from the control at 12.5 and 100 % of elutriate [$F_{(5,12)} = 8.9$; $P < 0.05$] (fig. II.2). On the other hand, the only significant inhibitory effect recorded for this species, when subjected to elutriate of RP (RP-E) was at 12.5% of elutriate [$F_{(5,12)} = 5.8$; $P < 0.05$], while in dilutions up to 100%, a stimulatory effect, though not significant, tended to occur (fig. II.2). Thereby, the percentage of inhibition in RP-E reached values minor or equal to 0%, being the $IC_{20} < 12.5\%$ (table II.3). On the contrary, for *C. vulgaris*, the percentage of inhibition in RP-E was mostly near 0% ($IC_{20} > 100\%$; fig. II.3, table II.3), suggesting that this sample did not significantly affect its growth rate [$F_{(5,12)} = 2.6$; $P > 0.05$; $IC_{20} > 100\%$]. In spite of this, 100% of VE-E induced a significant inhibition of *C. vulgaris* growth [$F_{(5,12)} = 14.2$; $P < 0.05$; $IC_{20} = 98\%$].

Table II.3 - NOEC and LOEC (% of water or elutriate) obtained for growth rate data, and $IC_{20}$ (% of water and elutriate) values determined to the percentage of inhibition, for *P. subcapitata* and *C. vulgaris* exposed to water samples and elutriates from VE and RP.

| Species | Sites | GR | | % I |
|---|---|---|---|---|
| | | NOEC | LOEC | $IC_{20}$ |
| *P. subcapitata* | VE-W | < 12.5 | ≤ 12.5 | 29.9 |
| | RP-W | < 12.5 | ≤ 12.5 | 58.8 |
| | VE-E | a | a | 73.6 |
| | RP-E | a | a | < 12.5 |
| *C. vulgaris* | VE-W | < 12.5 | ≤ 12.5 | a |
| | RP-W | a | a | a |
| | VE-E | > 100 | ≥ 100 | 98 |
| | RP-E | > 100 | ≥ 100 | > 100 |

GR = growth rate; %I = percentage of inhibition; NOEC = no-observed effect concentration; LOEC = lowest-observed effect concentration; $IC_{20}$ = concentration that induced 20% of inhibition; VE-W = water from VE; RP-W = water from RP; VE-E = elutriate from VE; RP-E = elutriate from RP; *a* = not obtainable.

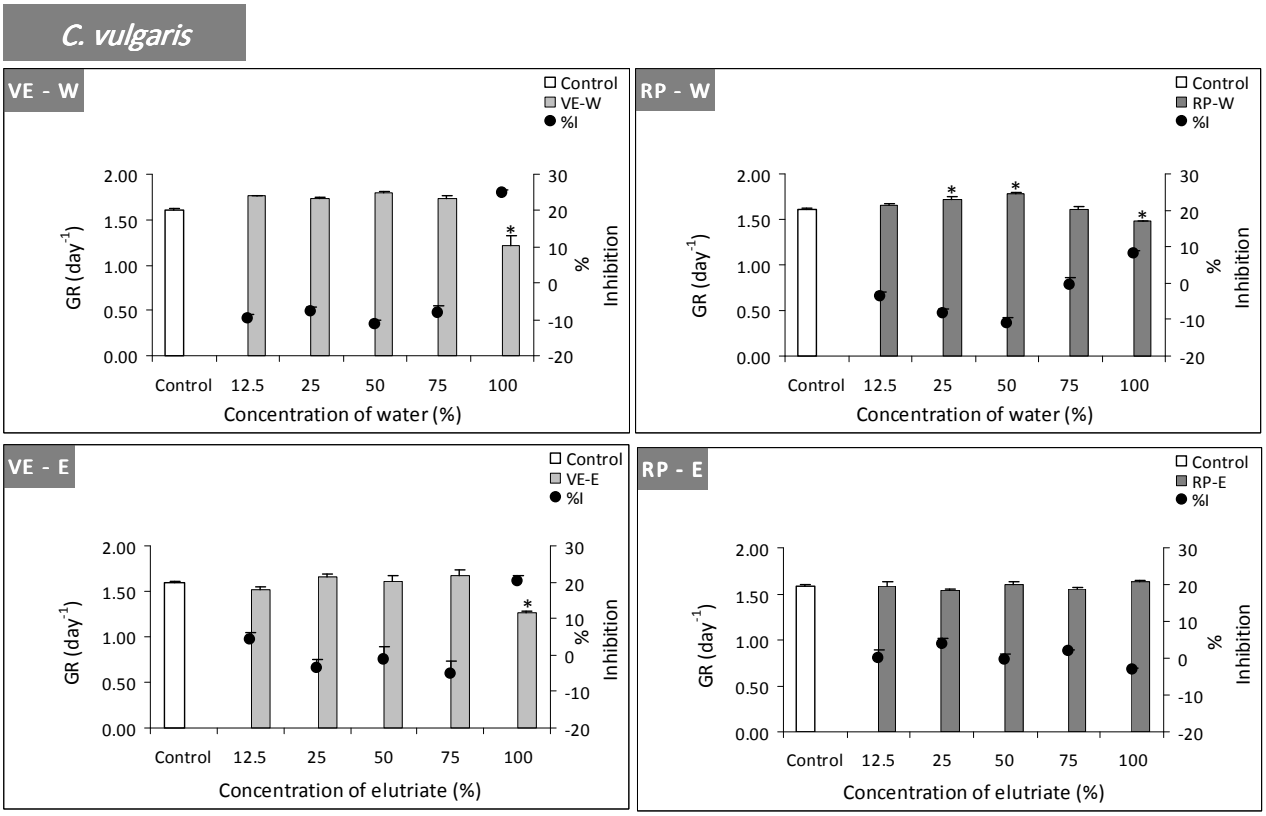


Figure II.3 - Growth rates (*GR*, $day^{-1}$) and % of inhibition (*%I*) for *C. vulgaris* exposed to water samples and elutriates from VE (*VE-W* and *VE-E*, respectively) and RP (*RP-W* and *RP-E*, respectively). Error bars represent standard deviation and the asterisks indicate a value significantly different from the control $P < 0.05$.

## 2.4 Discussion

The input of nutrients deriving from the dispersion of fertilisers on rice and other crop cultures in the vicinity of River Pranto, from point discharges emitted by husbandry areas upstream the rice paddy, and from agricultural run-off of the rice fields and leaching processes, led to low levels of organic contamination, in the surrounding aquatic ecosystem of Quinta do Seminário rice fields.

According to Chapman (1997), in most natural surface waters, phosphorus ranges from 0.005 to 0.020 mg $L^{-1}$ $PO_4^{3-}$, with concentrations as low as 0.001 mg $L^{-1}$ $PO_4^{3-}$ found in pristine waters. Overall, in this study, water samples exhibited phosphate values above that range, especially from RP-W (table II.1). On the other hand, pH and the nitrogen compounds determined in water samples were below the maximum recommended values for the quality of irrigation and surface waters in Portugal (MA 1998). Yet, concentrations of nitrate in lakes exceeding 0.2 mg $L^{-1}$ $NO_3^-$ -N, as measured in RP-W, may indicate possible eutrophic conditions (Chapman 1997). In opposition to water samples, elutriates present a more conspicuous content of nutrients, probably due to the nutrient contribution coming from MBL used in their preparation, plus the organic compounds that deadsorved from the sediment particles.

The qualitative chemical analyses did not reveal the presence of the pesticides applied in rice paddies. For instance, molinate and propanil, the active ingredients of the most applied herbicides in rice fields (at a rate of 40 - 60 kg $ha^{-1}$ for molinate, and two applications of 7 L propanil $ha^{-1}$ each, with three-days spacing between them) are readily dissipated and degraded. Previous studies reported half-lives between 40 - 160 days for molinate in flooded soil (Tomlin 2000) but of 60.8 and 62.4 days in lake and river water, respectively (Konstantinou et al. 2001); whilst for propanil they were of 55.4 and 60.3 days in river and lake waters (Konstantinou et al. 2001), respectively. Castro et al. (2005) revealed that the highest molinate concentrations in surface waters were observed in the day of Ordram® application (in April-May) and following days, decreasing throughout summer and becoming undetectable during winter. Similar results, though in different water drainage systems, were attained by Albanis et al. (1998), Jiménez et al. (1999), Santos et al. (2000) and Okamura et al. (2002). Cerejeira et al. (2003) reinforced the drastic decrease of molinate concentration in the beginning of August. Santos et al. (2000) and Ferraz et al. (2004) refer to analogous persistence times for propanil, what was attributed to several biotic and abiotic processes. Although the half-lives of the target pesticides are relatively reduced, the adsorption of these chemicals or their degradation products to soil particles could still represent potential impairments in the aquatic sensitive trophic levels, deriving from the field runoff/drainage. For this reason, it was important to perform the sampling during August, simultaneously to the drainage of fields.

With respect to bioassays *C. vulgaris* was in general more tolerant to water samples and elutriates from VE and RP than *P. subcapitata*. This is specially perceived under water samples, which encompassed more significant growth rate responses with antagonist trends between species. Even though, under sediment elutriates, the $IC_{20}$ for VE-E and RP-E attained lower values for *P. subcapitata* (73.6 and < 12.5%, respectively) than for *C. vulgaris* (98 and > 100%, respectively) (table II.3), relatively to their respective controls. In fact, Nyholm and Källqvist (1989), Blaise and Ménard (1998) and Ma et al. (2004) suggested that *Chlorella* species are not as sensitive as *Pseudokirchneriella*. Nevertheless, there are other studies where *Pseudokirchneriella* was not the most sensitive species (de Figueiredo et al. 2004, Pereira et al. 2005). Thus, the best strategy to perform algal growth inhibition tests and conclude about the toxicity of a sample is to use more than one algae.

Xenobiotics determined in water samples from VE and RP may exert harmful effects upon *P. subcapitata* growth, as significant inhibitory percentages were obtained under every dilution tested of VE-W and RP-W. Furthermore, results indicated an apparently stronger growth inhibition promoted by VE-W to *P. subcapitata* than RP-W, which is reflected by its lower $IC_{20}$ (table II.3). Actually, it is quite difficult to discern possible cause-effect relationships, as various effects including the synergistic, antagonistic, and additive effects of all the chemical, physical and biological components present in complex mixtures can adversely affect the physiological and biochemical functions of the test organisms (Pardos et al. 1998).

On its side, the growth of *C. vulgaris* was only significantly inhibited to 100% of VE-W and RP-W, whilst for intermediate dilutions the enhancement of its growth rates occurred, being statistically higher than those of the control for 25 and 50% of RP-W. This pattern may traduce an hormetic response, which has already been widely documented as a common dose-response relationship (Calabrese 2002), including in studies with WET test organisms (Delistraty and Yokel 1999, Chapman 2000). The prevalence of the stimulating effect in lower dilutions could be assigned to the nutrients provided in the sample and in the MBL, since previously published results (de Figueiredo et al. 2004, Gonçalves et al. 2005) proved that, for both *P. subcapitata* and *C. vulgaris*, growth in diluted MBL with up to 40% distilled water is not significantly affected in relation to growth under nutrient saturated conditions (MBL) but above 50% a significant growth reduction may be observed for *C. vulgaris*. Actually, the observed growth stimulation of *C. vulgaris* could be assigned to the toxicity masking effect of nutrients present in field water samples and MBL, which, in turn, for *P. subcapitata* is not sufficiently strong to prevent its growth impairment. This highlights, once more, the need for using more than one algal species in this kind of growth inhibition assays due to specific sensitivity variation. In contrast, the algal growth inhibition under 100% of VE-W and RP-W should be mainly due to nutrient deficiency even if a toxicant effect is

also associated. Olguín et al. (2004) strengthened the positive effect of nutrients on algal biomass, once the spatial and temporal variations displayed by the bioassays with a river water, for *Chlorella pyrenoidosa* and *Scenedesmus acutus*, paralleled the variations in the major nutrients (determined as the concentration of ammonium and orthophosphate ions). Therefore, they suggested the incidence of a stimulating effect of nutrients over the inhibiting effect of toxicants in water samples.

Generally, the more noticeable effects underwent either by *P. subcapitata* or *C. vulgaris* arose when subjected to water samples than under sediment elutriates, which is in relative compliance with the absence of chemicals on sediment and with the fact that elutriates presented higher nutrient contents. For *C. vulgaris*, the percentage of inhibition in RP-E was mostly near 0%, suggesting that this sample did not significantly affect its growth rate. On a whole, elutriates from VE-E brought about some more significantly different responses from the control, in comparison with RP-E, for both green algal species.

The pesticides introduced in rice fields do not seem to induce chronic effects on aquatic organisms due to their quick degradation but, instead, they may tend to be hazardous during acute exposures.

Overall, one should kept in mind that WET tests may provide an uncertain level of protection, since they are developed under controlled conditions in laboratory, meaning that WET tests do not account with the action of quite a few processes occurring under field exposures. However, WET tests are important predictive tools in a screening phase of hazard identification (Chapman 2000), as became explicit in the present work.

## 2.5 Conclusions

Agricultural practices undertaken in the River Pranto catchment basin and, mainly, in Quinta do Seminário rice fields, led to certain levels of organic contamination, reflected by phosphate values in water samples from VE and RP, which can represent a potential situation of eutrophication. Chemical analyses proved the occurrence of chemical compounds only in water samples from both sites, though any of those corresponded to pesticides applied in the rice culture, what was assigned to their short half-lives.

In general, bioassays reflected that *P. subcapitata* was more sensitive than *C. vulgaris*. Under water samples from VE and RP, *P. subcapitata* growth was significantly inhibited by any water dilution, probably due to the presence of xenobiotics. On its side, *C. vulgaris* exposed to RP-W exhibited an hormetic response. Elutriates were less toxic to both algae species. Overall, water samples, mainly those from the main irrigation/drainage canal (VE-W) of the rice fields, were

more deleterious to microalgae than those from RP or any of elutriates. However, we suggest that the use of more than one microalgae species, from different phytoplanktonic groups, in growth inhibition tests should be conducted, since algal specific sensitivity may strongly vary.

The agri-environmental measures implemented in this local farming area, namely regarding the use of agrochemicals, should be more rigorously followed as a way to protect the aquatic biodiversity. The generated data mainly evidence the growth impairment of a sensitive microalgae species for a low protection level. Therefore, we suggest a frequent monitoring of water quality, paralleled by the improvement of good agricultural practices.

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# Chapter III

## The individual and mixture toxicity effects of MIKADO® and VIPER® on two trophic levels

# The individual and mixture toxicity effects of MIKADO® and VIPER® on two trophic levels

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**Sumitted to |** Aquatic Toxicology

## Abstract

The toxicity of single and combined formulated herbicides (Mikado® and Viper®) was assessed on several endpoints in species from two trophic levels: algae growth – *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* –, immobilisation and life-history traits (only for single compound toxicity) of daphnids – *Daphnia longispina* and *Daphnia magna*. Viper was the most toxic formulated herbicide. It was hypothesized that the toxicity of both formulated herbicides could have been enhanced due to the presence of adjuvants, especially for Viper. In most cases, the sub-lethal endpoints were the most responsive and affected by both formulations, comparatively to their acute effects. Concentration addition (CA) and independent action (IA) models provided an accurate description of Mikado and Viper joint action on algae growth and immobilisation of daphnids, though significant deviations were always detected. A low-dose antagonism and high-dose synergism was identified for *P. subcapitata*, whereas *C. vulgaris* response deviated antagonistically from CA and synergistically from IA. For both daphnids, however, synergistic effects were observed for higher mixture concentrations. Under a regulatory standpoint, CA provided the most conservative estimation either because the mixture effects were overestimated or less sub-estimated than IA. Overall, the great sensitivity differences observed within species did not allow the conclusion that one trophic level was more tolerant than the other. Instead, *P. subcapitata* was always the most sensitive species to both herbicide formulations, followed by *D. longispina*, whilst *D. magna* and *C. vulgaris* were the most tolerant species. On a whole, further studies are needed towards a comprehensive understanding of herbicides mode of action, their effects at lower biological-level endpoints, and under different mixture designs.

**Key-words:** Herbicides, acute and chronic effects, green microalgae, standard and autochthonous daphnids, mixture toxicity, concentration addition model, independent action model

## 3.1 Introduction

Herbicides are the major type of pesticides applied [in 2002-03 they represented ca. 35-38% of the pesticides used in Europe (ECPA 2003, EC 2007)], especially in areas of intensive agricultural production. As a result, herbicides can easily reach the surrounding aquatic systems through spray drift, run-off and/or soil percolation from agricultural catchment basins. Moreover, they frequently occur as complex mixtures that represent an environmental concern towards the integrity of aquatic systems (Cedergreen and Streibig 2005, Junghans et al. 2006, Relyea and Hoverman 2006, Bonnet et al. 2008).

Although herbicides are designed to control plants they can also affect the fitness of aquatic animals, whenever there is a similar biological target site of action pre-empting a cascade of biochemical and physiological impairments. An additional concern related with herbicide impacts on non-target individuals arises when dealing with formulated products. The addition of adjuvants to pesticide formulations intends to enhance the effectiveness of the active ingredient (a.i.) by improving its uptake, persistence, distribution and behaviour in the environment (Cox and Surgan 2006, Cedergreen et al. 2007). Therefore, due to their biological and chemically active nature, adjuvants may also enhance the toxicity of the a.i. to non-target organisms (Stark and Walthall 2003, Cox and Surgan 2006, Cedergreen et al. 2007). Although European guidance documents (EC 2002a, 2002b) and regulations establishing the ecotoxicological assessment of pesticides under their registration process (EEC 1991) recommend the testing of formulations, their ecotoxicological data are seemingly unpublished since the available studies in open literature mainly accomplish the evaluation of the respective a.i.s (Cox and Surgan 2006).

Furthermore, the great majority of risk assessments (RA) and regulatory requirements are not yet devoted to ecotoxicological information of multiple chemical mixtures and stressors in the aquatic ecosystems (Cassee et al. 1998, ECETOC 2001, EC 2003). Understanding mixture effects is a challenge for ecotoxicologists and regulators as it may represent a step forward into the management and conservation of freshwater ecosystems (ECETOC 2001, Relyea and Hoverman 2006). Even though, the number of studies evaluating the toxicity of pesticide mixtures, namely those composed by herbicides (*e.g.*, Cedergreen and Streibig 2005, Relyea and Hoverman 2006, Cedergreen et al. 2007, Schuler and Rand 2008) has been recently growing, the available guidelines concerning mixture toxicity assessment (*e.g.*, U.S.EPA 2000, ATSDR 2001) are still under improvement (McCarty and Borgert 2006).

Considering that is unpractical to test all possible mixture combinations occurring in the environment, due to a great temporal and spatial variation of chemical concentrations, model approaches may be used to predict mixture hazard. The two conceptual models mostly applied

are concentration addition (CA) and independent action (IA), which predict mixture toxicity based on known toxicities of similarly- and dissimilarly-acting individual toxicants, respectively (Backhaus et al. 2000, Syberg et al. 2008). CA describes the mixture toxicity of compounds that interact on a common target site pre-empting similar mechanisms of action, hence it is expected that different compounds induce a similar toxicological effect (Faust et al. 2003). In turn, IA postulates independent and different target sites and mechanisms of action addressed by single toxicants that will lead to a common toxicological effect through distinct chains and reactions within an organism (Faust et al. 2003, Barata et al. 2006). A common characteristic is that both models assume non-interaction between toxicants, being their joint action additive. Any deviation from their reference prediction indicates interaction (synergistic – more than additive action – or antagonistic - less than additive action) (Syberg et al. 2008).

The evaluation of individual or combined herbicide toxicity in the aquatic system had been performed through the use of test organisms belonging to different trophic levels, ranging from microorganisms (*e.g.*, Bonnet et al. 2008, Cedergreen et al. 2006), algae (*e.g.*, Ma et al. 2002, Junghans et al. 2003), macrophytes (*e.g.*, Michel et al. 2004, Cedergreen et al. 2007), invertebrates (*e.g.*, Villaroel et al. 2003, Banks et al. 2005) up to fish (*e.g.*, Kreutz et al. 2008, Fatima et al. 2007). Though algae are usually very sensitive species to herbicide effects, daphnids have also been considered sensitive test organisms to these chemicals (EC 2002b). In fact, algae and daphnids belong to two basic trophic levels – producers and primary consumers, respectively – that sustain and allow energy transfer along freshwater trophic chains (*e.g.*, Källqvist and Romstad 1994, Hanazato 2001). As such, any impairments occurring on their fitness due to chemical exposures may constrain the maintenance of natural populations, which in turn may induce bottom-up and top-down adverse ecological effects (Källqvist and Romstad 1994, Allen et al. 1995, Relyea and Hoverman 2006).

Considering what was said above, the aim of the present study was to (1) determine the acute and/or sub-lethal effects of Mikado and Viper on two trophic levels - green microalgae (*Pseudokirchneriella subcapitata* and *Chlorella vulgaris*) and daphnids (*Daphnia longispina* and *Daphnia magna*), (2) evaluate the toxicity of binary mixtures of Mikado® and Viper® (hereinafter referred as Mikado and Viper) on microalgae growth and daphnids' immobilisation by fitting CA and IA models to our data, (3) compare the sensitivity of the assessed endpoints and trophic levels used.

This work makes part of a more comprehensive study, concerning an agricultural area intensively exploited for corn and rice production, in which Mikado and Viper are the herbicides respectively used to control weeds. They are usually applied more than one time during the same crop season due to the extension of the cropping area that has to be sprayed. Mikado and Viper

are relatively new herbicides in the European market (Meazza et al. 2002, Bird et al. 2006) and the related available ecotoxicological studies are scarce, as far as authors are aware. Therefore, the final outcome of this work may complement the available ecotoxicological data, which is essential to model pesticide effects in tandem with species sensitivity distributions. Under an ERA perspective, the development of such information may allow the estimation and characterisation of potential hazards and risks following application of those formulated herbicides, what in turn may help on risk assessors' decisions towards the mitigation of ecological problems.

## 3.2 Material and methods

The toxicity of the single herbicidal compounds Mikado and Viper and their mixture was experimentally analysed in a growth assay with microalgae (*P. subcapitata* and *C. vulgaris*), and in acute immobilisation and chronic (only for single compound toxicity) assays with daphnids (*D. longispina* and *D. magna*).

### 3.2.1 Test organisms

*P. subcapitata* Korshikov (Hindak) and *C. vulgaris* Beijerinck were maintained in unialgal batch cultures with Woods Hole MBL medium (referred as MBL), at 20 ± 2º C and $16^{L}$:$8^{D}$ h photoperiod. New cultures were initiated with algae harvested from cultures at the exponential growth phase (*i.e.*, 5-7 days-old) and then inoculated into fresh medium.

Monoclonal bulk cultures of *D. longispina* [clone EM7, *sensu* Antunes et al. (2003), isolated from a population collected in Lake Vela, and maintained for several generations in the laboratory] and *D. magna* [clone A, *sensu* Baird et al. (1989a)] were reared in ASTM (ASTM 1980) enriched with a standard organic additive (*Ascophylum nudosum* seaweed extract; Baird et al. 1989b), under 20 ± 2 °C and a $16^{L}$:$8^{D}$ h photoperiod. Cultures were renewed and fed (with *P. subcapitata* at a rate of 1.50 and 3.00 x $10^{5}$ cells $mL^{-1}$ *Daphnia*$^{-1}$ for *D. longispina* and *D. magna*, respectively) every other day.

### 3.2.2 Chemicals

Mikado, marketed in Europe by Bayer Crop Science, is a systemic foliar-applied post-emergence herbicide mostly used in corn crops through terrestrial application, for the control of broadleaf weeds and annual grasses (Matringe et al. 2005). Mikado is produced as a concentrated suspension containing 300 g a.i. $L^{-1}$, being its recommended rate of application of 1.5 - 2 L $ha^{-1}$. Its a.i. is sulcotrione, a 2-benzoylcyclohexanodione from the triketone class of compounds, which mode of action (m.o.a.) relies on the inhibition of the enzyme 4-hydroxyphenylpyruvate

dioxygenase (HPPD) (Matringe et al. 2005). In plants, HPPD is involved in the catabolism of tyrosine and consequent biosynthesis of α-tocopherol and plastoquinones. Plastoquinones are vital components of the chloroplastic electron-transfer chain of photosystem II (PSII) and, on the other hand, they constitute critical cofactors for phytoene desaturase, which is involved in the biosynthesis of carotenoid pigments. Concomitantly, the decrease of α-tocopherol also affects PSII. As such, plastoquinone depletion due to HPPD inhibition will disrupt the carotenoid biosynthesis, leading to the destabilisation of the photosynthetic apparatus that is in turn sustained by those pigments. This situation will enable necrosis under strong light intensity and loss of chlorophyll, causing plant bleaching and death (Meazza et al. 2002, Shaner 2003, Matringe et al. 2005, Abendroth et al. 2006).

Viper (DOW AgroSciences) is also a post-emergence systemic herbicide, though it is applied in rice fields via terrestrial or aerial spraying, for the control of annual grasses, sedges, and broadleaf weeds (Roberts et al. 2003). Its formulation is oil dispersible, containing 97.86% of other ingredients, including an adjuvant that has methanol (DOW AgroSciences 2006). Viper is applied at a rate 2 - 2.5 L $ha^{-1}$. The a.i. of Viper is penoxsulam ([20.4 g a.i. $L^{-1}$), a triazolopyrimidine sulfonamide compound, which acts as an acetolactate synthase (ALS; now known as acetohydroxyacid synthase, AHAS) inhibitor. ALS targets the biosynthesis of branch-chained aminoacids (valine, leucine, isoleucine), a metabolic pathway found in fungi, microorganisms and plants, but not in animals (Roberts et al. 2003). The inhibition of ALS may occur at low-use rates of the specific herbicides, causing the decrease of aminoacid and protein synthesis, resulting in a rapid cessation of organism growth. According to WHO (2005), penoxsulam is unlikely to present acute hazard for non-target organisms under normal use.

The test concentrations of both formulated herbicides were calculated in terms of their a.i. concentration into the respective product and expressed as mg a.i. $L^{-1}$. The tested concentration ranges for single compound or mixture testing were settled by geometric dilutions of a concentrated stock solution, which was prepared through the dilution of the respective formulated product with distilled water, before test beginning and/or renewal.

### 3.2.3 Toxicity of individual compounds

For each individual component of the mixture, the complete concentration-response relationship had to be obtained over a range from 1 to at least 80% effect (Backhaus et al. 2000).

*a. Microalgae growth assay*

Algae growth assay was conducted according to OECD (2002) guidelines, following static testing conditions. Three replicates were exposed to different nominal concentration ranges of

Mikado (*P. subcapitata*: 0.39, 0.78, 1.56, 3.12, 6.25, 12.50 mg sulcotrione $L^{-1}$; *C. vulgaris*: 198.9, 228.7, 263.0, 347.5, 347.8, 400.0 mg sulcotrione $L^{-1}$) and Viper (*P. subcapitata*: 0.015, 0.031, 0.061, 0.12, 0.24, 0.49 mg penoxsulam $L^{-1}$; *C. vulgaris*: 0.12, 0.24, 0.49, 0.98, 1.95 mg penoxsulam $L^{-1}$) in 100 mL glass vials containing 40 mL of control (MBL) or test solution (Gonçalves et al. 2005). Initial cell densities were approximately $10^5$ cells $mL^{-1}$. The test was run under constant agitation (≈ 100 rpm in an orbital shaker) during 96h, in the same conditions of algal cultures. Algae growth was evaluated through cell density determination (counting of cells on a microscope Olympus CKX41 using a Neubauer chamber) expressed as % of the control.

*b. Acute and chronic assays with daphnids*

The 48-h acute exposures followed the procedures established by OECD (2004) for static testing conditions. Five neonates (< 24 h old, from the 3rd to 6th brood) were randomly assigned per vessel, in a total of four replicates per treatment. The test was carried out in 100 mL vials with 50 mL of control (ASTM) or test solution, under the same conditions mentioned for the rearing of daphnids, except that no food or organic additives were supplemented. The nominal concentrations tested for Mikado were 192.9, 231.5, 277.8, 333.3, 400.0 mg sulcotrione $L^{-1}$ for *D. longispina* and 327.7, 409.6, 512.0, 640.0, 800.0 mg sulcotrione $L^{-1}$ for *D. magna*; whereas Viper was tested at 0.004, 0.008, 0.016, 0.031, 0.063, 0.12, 0.25, 0.50, 1.0 mg penoxsulam $L^{-1}$ for *D. longispina* and 0.23, 0.47, 0.94, 1.87, 3.75, 7.50 mg penoxsulam $L^{-1}$ for *D. magna*. After the exposure period each vial was monitored for immobilised neonates.

The chronic reproduction assay with daphnids (OECD 1998) was carried out during 21 days on a semi-static test design, being renewed to newly-prepared test solutions every other day. Ten individual replicates of newborn daphnids (< 24-h old, from the 3rd to 6th brood) were exposed (in 50 mL glass vials) to a range of nominal concentrations for Mikado (*D. longispina*: 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 mg sulcotrione $L^{-1}$; *D. magna*: 10.6, 17.5, 26.9, 47.7, 78.8, 130.0 mg sulcotrione $L^{-1}$) and Viper (*D. longispina*: 0.004, 0.006, 0.012, 0.016, 0.026, 0.042 mg penoxsulam $L^{-1}$; *D. magna*: 0.006, 0.011, 0.018, 0.031, 0.052, 0.088, 0.150 mg penoxsulam $L^{-1}$) plus the control (ASTM). The test conditions were the same already described for the maintenance of daphnids, except that they were fed at least five days per week, with their respective *P. subcapitata* ratio (see above). Animals were daily observed for mortality and offspring production, being the neonates counted and discarded. The endpoints recorded were fecundity (reproductive output) the age at first reproduction (AFR) and the somatic growth rate (SGR) of parent females, which was estimated from (Burns 2000):

$$SGR = [\ln(l_f) - \ln(l_i)] / \Delta t$$

where Δt is the testing interval period in days, $l_f$ and $l_i$ are, respectively, the final and initial body lengths estimated from the moult exopodite measure, according to the allometric relations publish by Pereira et al. (2004). Additionally, the value of *r* (rate of population increase) was derived from the Euler-Lotka equation (Meyer et al. 1986), which integrates survival and fecundity estimates:

$$\sum e^{-r.x} . l_x . m_x = 1$$

where *x* is the age class (days; 0…*n*), $l_x$ is the probability of surviving at age *x*, and $m_x$ is fecundity at age *x.* The standard deviation was determined according to Jackknife technique (Meyer et al. 1986).

### 3.2.4 Mixture toxicity

The toxicity of a binary mixture was determined using a fixed ratio design. It means that within the mixture, the concentration ratio of the individual compounds was kept constant, but the whole mixture concentration gradually changed (Backhaus et al. 2000). One mixture ratio was examined, in which each component in the mixture was present at the same ratio of its own individual $EC_{50}$s values [thereby called an equitoxic mixture (Backhaus et al. 2000)] according to $TU_i = (c_i / EC_{50i})$, where $TU_i$ is the relative strength of the compound *i* in the mixture, $c_i$ is the concentration of compound *i* in the mixture, and $EC_{50i}$ is the concentration of the individual compound *i* inducing 50% of toxic effect.

Considering this, different stock equitoxic mixtures were prepared, based on the $EC_{50}$s previously determined for microalgae growth assays and daphnid immobilisation tests with Mikado and Viper (table III.3). These concentrated stock solutions were then diluted in a geometric series through the introduction of the correct volumes into the correspondent glass vessels, to obtain the final mixture test solutions (Warne 2003). Afterwards, the toxicity tests for algae reproduction and daphnids 48-h immobilisation were carried out using the same procedures and conditions outlined for single substances (*c.f.*, section 3.2.3). The mixture concentrations were defined to allow the toxicity range from 10 to at least 90% effect. For *P. subcapitata* nominal concentrations varied between 0.57 and 3.15 mg sulcotrione $L^{-1}$ for Mikado and 0.014 and 0.078 mg penoxsulam $L^{-1}$ for Viper, whereas for *C. vulgaris* they were within 92.21 and 281.40 mg sulcotrione $L^{-1}$ for Mikado and 0.22 and 0.68 mg penoxsulam $L^{-1}$ for Viper. For *D. longispina*, the tested concentrations varied between 10.99 and 42.54 mg sulcotrione $L^{-1}$ for Mikado and 0.0044 and 0.021 mg penoxsulam $L^{-1}$ for Viper, while for *D. magna* the concentration ranges were from 15.18 to 114.28 mg sulcotrione $L^{-1}$ for Mikado and from 0.064 to 0.49 mg penoxsulam $L^{-1}$ for Viper.

### 3.2.5 Data analysis

For individual compound toxicity, the $EC_{50}$ point estimates and respective confidence limits at 95% (95%-CL) were calculated for the growth of microalgae (96-h $EC_{50}$; % cell density), the acute immobilisation of daphnids (48-h $EC_{50}$; number of immobilised daphnids) and the fecundity of daphnids obtained upon chronic exposures (21-d $EC_{50}$; average number of offspring, *i.e.*, fecundity), using Probit analyses (Finney 1971). The significance of Mikado and Viper individual effects on algae growth and each chronic endpoint monitored for daphnids was tested with a one-way ANOVA. Whenever a significant difference ($P < 0.05$) was found, the LOEC (low-observed-effect concentration) values were determined using Dunnett's test for multiple comparisons of each individual concentration with the control, *per* treatment (Zar 1996).

For mixture toxicity, the $EC_{50}$ point estimate and respective confidence limits at 95% (95%-CL) were calculated for the nominal concentrations of Mikado and Viper used in the mixture, both for the growth of microalgae (96-h $EC_{50}$; % cell density) and the acute immobilisation of daphnids (48-h $EC_{50}$; number of immobilised daphnids), using again a Probit analyses (Finney 1971).

Additionally, in order to evaluate the mixture effects on algae growth and daphnid survival (immobilisation) both CA and IA models were fitted to the experimental data, since as long as authors are aware, the m.o.a.s of Mikado and Viper a.i.s are not fully addressed to both groups of organisms. Thereby, it was not possible to choose one single model based on the limited m.o.a. information.

The mathematical expression defining CA is $\sum (c_i / EC_{xi}) = 1$, where $c_i$ is the concentration of compound *i* in the mixture, $EC_{xi}$ is the concentration of the compound *i* that provokes *x*% effect when individually tested. The fraction $c_i / EC_{xi}$ represents the concentration of the *i*th compound scaled for its respective single toxicity and is called as the toxic unit of compound *i* (Backhaus et al. 2000). The dimensionless TU had its origin, indeed, in the CA concept, according to which any component in the mixture may be replaced by another similarly acting chemical without changing the overall mixture toxicity, as long as the correspondent TU is maintained, as to obtain a TU summation equalling 1 (Junghans et al. 2006).

The mathematical formulation used for IA concept was: $Y = \mu_{max} \prod q_i (c_i)$, where *Y* is the biological response, $\mu_{max}$ is the control response for the endpoint analysed, $\prod$ is the multiplication function, $c_i$ is the concentration of compound *i* in the mixture and $q_i(c_i)$ is the probability of non-response function.

CA and IA were applied to experimental data according to the procedures described and developed by Jonker et al. (2005). Deviations of observed data from the reference models (through addition of parameters *a* and/or *b* to the reference models) were also ascertained for their significance and pattern type, being analysed only two of the biologically relevant deviation

models – synergism/antagonism (S/A) and dose level-dependent (DL; *i.e.*, the deviation from the reference models at low dose levels is different from that at high dose levels) deviations – since our experimental design considered just one mixture ratio (precluding a feasible fit of a potential dose ratio-dependent deviation). Considering this, four main steps were pursued to address the mixture toxicity on algae growth and daphnid immobilisation endpoints, for each tested species. First, a single log-logistic dose-response curve was determined for each data set obtained with individual herbicide toxicities, in order to get starting values for the parameters that feed the following mixture models. Secondly, the reference models CA and IA or their respective deviation models were fitted to the individual herbicide concentration-response curves by a maximum likelihood method (the applied likelihood function was the log-logistic function). Either for CA or IA and their deviations, the fitting process was conducted through a series of iteractions performed in a spreadsheet environment by the built-in solver function (initially fed with data obtained from individual compound toxicities – individual $EC_{50}$s and $\mu_{max}$ – and slopes – determined in the first step), which goal is to minimise the sum of squared residuals (SS; for continuous data like growth) or data likelihood (L; for binary endpoints like survival). The third step concerns the comparison of fits (of CA and IA models and their deviations) through calculation of the likelihood ratio statistic ($\chi^2$). The fourth step entails the biological interpretation of the parameters (according to table III.1) of the significant deviation model that best described mixture effects trends, for the different endpoints and species considered (Jonker et al. 2005). The outcome of DL deviation is presented for each data set, except when no significant fit was achieved relatively to the reference or S/A deviation models.

Table III.1 - Meaning of the parameters (*a* and *b*) added to CA (concentration addition) and IA (independent action) reference models, as to define two deviation types: S/A – synergism/antagonism deviation, DL – dose-level deviation.

| | Parameter value | Deviation pattern | |
|---|---|---|---|
| | | S/A | DL |
| Parameter *a* | a>0 | antagonism | antagonism at low dose level and synergism at high dose level |
| (CA or IA) | a<0 | synergism | synergism at low dose level and antagonism at high dose level |
| Parameter *b* | b>1 | - | change at lower dose level than $EC_{50}$ |
| (CA) | b=1 | - | change at the $EC_{50}$ |
| | 0<b<1 | - | change at high dose level than $EC_{50}$ |
| | b<0 | - | no change; the magnitude of S/A is dose level dependent |
| (IA) | b>2 | - | change at lower dose level than $EC_{50}$ |
| | b=2 | - | change at the $EC_{50}$ |
| | 1<b<2 | - | change at high dose level than $EC_{50}$ |
| | b<1 | - | no change; the magnitude of S/A is effect level dependent |

## 3.3 Results

All ecotoxicological tests fulfilled the validity requirements established on their respective OECD (1998, 2002, 2004) guidelines.

### 3.3.1 Single compound toxicity

*a. Microalgae growth assay*

A positive dose-response relationship was obtained for both microalgae species either exposed to Mikado or Viper (fig. III.1). However, Mikado was less toxic than Viper, what is immediately perceived from the lower testing concentrations, but also because the determined point estimates were two to three orders of magnitude higher than those achieved for the latter herbicide (tables III.2, III.3). Thus, Mikado was relatively toxic to *P. subcapitata* growth (96-h $EC_{50}$=1.58 mg $L^{-1}$, LOEC=1.56 mg $L^{-1}$), whilst for *C. vulgaris* its toxicity was quite reduced (96-h $EC_{50}$=281.40 mg $L^{-1}$, LOEC=302.46 mg $L^{-1}$). In turn, Viper had greatly impaired *P. subcapitata* growth (96-h $EC_{50}$=0.039 mg $L^{-1}$, LOEC=0.061 mg $L^{-1}$), while *C. vulgaris* was less affected by it, since higher 96-h $EC_{50}$ and LOEC values (0.68 and 0.49 mg $L^{-1}$, respectively) were obtained. Above all, it is noticeable that *P. subcapitata* was more sensitive than *C. vulgaris*, independently of the formulated product tested, given the lower values of its point estimates.

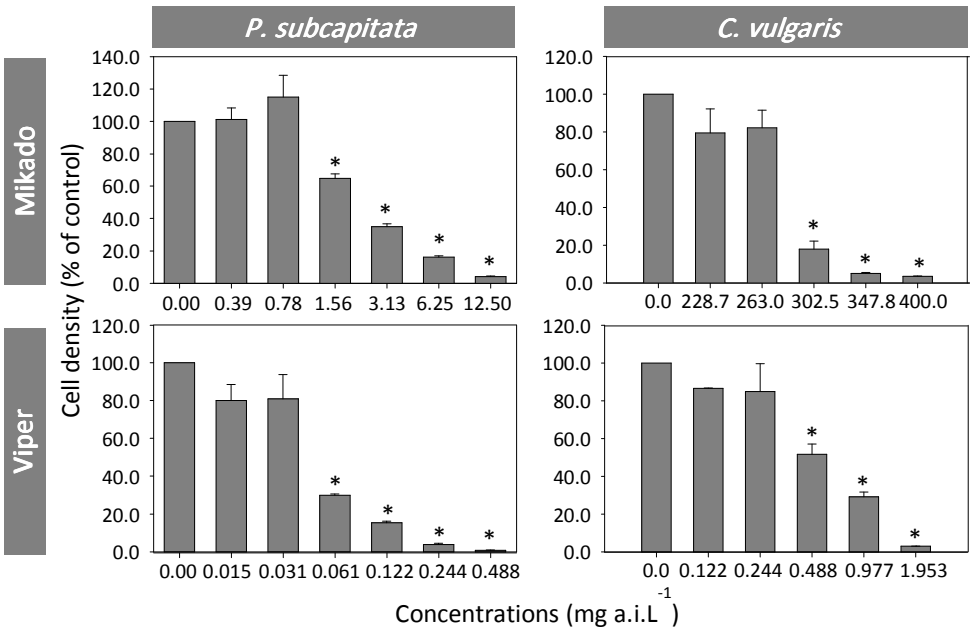


Figure III.1 - *P. subcapitata* and *C. vulgaris* growth (cell density expressed as % of control) along increasing concentrations of Mikado and Viper. Error bars represent standard deviation. Significant differences from the control are signed as * ($P \leq 0.05$).

Table III.2 - One-way ANOVA outcome summary and LOEC values ($P \leq 0.05$) (mg $L^{-1}$) for the growth of microalgae species and the chronic endpoints evaluated for the two daphnid species.

| | | | Mikado | | | | Viper | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Assay | Species | Endpoints | *F* | *d.f.* | *P* | LOEC | *F* | *d.f.* | *P* | LOEC |
| Sub-chronic | *P. subcapitata* | CD | 51.656 | 6,14 | <0.001 | 1.56 | 40.462 | 6,15 | <0.001 | 0.061 |
| | *C. vulgaris* | CD | 46.658 | 6,14 | <0.001 | 302.5 | 37.075 | 5,12 | <0.001 | 0.488 |
| Chronic | *D. longispina* | Fecundity | 40.936 | 6,54 | <0.001 | 10.0 | 21.483 | 6,52 | <0.001 | 0.016 |
| | | AFR | 42.444 | 6,54 | <0.001 | 40.0 | 41.326 | 6,52 | <0.011 | 0.026 |
| | | *r* | 48.329 | 6,60 | <0.001 | 20.0 | 60.588 | 6,55 | <0.001 | 0.016 |
| | | SGR | 256.028 | 6,54 | <0.001 | ≤2.5 | 10.525 | 6,52 | <0.001 | 0.010 |
| | *D. magna* | Fecundity | 43.401 | 6,61 | <0.001 | 17.5 | 43.324 | 6,55 | <0.001 | 0.052 |
| | | AFR | 3.305 | 6,61 | 0.007 | 130.0 | 6.676 | 6,55 | <0.001 | 0.088 |
| | | *r* | 7.386 | 6,63 | <0.001 | 130.0 | 29.338 | 7,72 | <0.001 | 0.052 |
| | | SGR | 25.520 | 6,61 | <0.001 | 17.5 | 18.079 | 6,55 | <0.001 | 0.011 |

ND – not determined, CD – cell density, AFR – age at first reproduction, *r* – intrinsic rate of population increase, SGR – somatic growth rate.

*b. Acute and chronic assays with daphnids*

Similarly, the impairments promoted by Mikado on acute and life-history traits of both daphnid species occurred at higher concentrations than those observed for Viper, indicating that it was less toxic. The 48-h $EC_{50}$ for *D. longispina* (262.2 mg $L^{-1}$) was about half of that estimated for *D. magna* (533.3 mg $L^{-1}$) undergoing Mikado exposure. Whereas more than one order of magnitude separated the 48-h $EC_{50}$ determined for *D. longispina* (0.11 mg $L^{-1}$) exposed to Viper from the one calculated for *D. magna* (2.27 mg $L^{-1}$) (table III.3).

For the chronic assays, the tested concentration ranges were generally one order of magnitude below the correspondent 48-h $EC_{50}$ value. Even though, a considerable mortality occurred during the reproduction assay for both species when exposed to higher concentrations of Viper [*D. longispina*: 80% mortality at 0.042 mg a.i. $L^{-1}$; *D. magna*: 60% at 0.052 mg a.i. $L^{-1}$ and 90% mortality at 0.15 mg a.i. $L^{-1}$]. The recorded mortality along the chronic testing of Mikado was always ≤ 20% for both cladocerans.

The reproductive output and SGR of daphnids were the most significantly impaired endpoints, declining with increasing concentrations of Mikado (fig. III.2, tables III.2, III.3). On the other hand, the endpoints AFR and *r* were less responsive to Mikado, showing higher LOEC values than fecundity and SGR for both species (fig. III.2, table III.2).

Table III.3 - $EC_{50}$ values (mg $L^{-1}$) and respective confidence limits at 95% (95%-CL) calculated for the parameters algae cell density (96-h $EC_{50}$), and the acute immobilisation (48-h $EC_{50}$) and fecundity (21-d $EC_{50}$) of daphnids, when subjected to the single compounds.

| | | **96-h $EC_{50}$** | | **48-h $EC_{50}$** | | **21-d $EC_{50}$** | |
|---|---|---|---|---|---|---|---|
| | Herbicide | *P. subcapitata* | *C. vulgaris* | *D. longispina* | *D. magna* | *D. longispina* | *D. magna* |
| **Single compound** | Mikado | 1.58 | 281.4 | 262.2 | 533.3 | 52.1 | 53.8 |
| | | (0.438-3.534) | (281.11-334.41) | (249.58-276.39) | (498.51-568.29) | (38.24-78.69) | (27.05-250.27) |
| | Viper | 0.039 | 0.68 | 0.11 | 2.27 | 0.028 | 0.093 |
| | | (0.022-0.067) | (0.434-1.101) | (0.024-0.285) | (1.839-2.883) | (0.0238-0.0334) | (0.0788-0.112) |
| **Mixture** | Mikado | 0.960 | 172.08 | 29.93 | 51.61 | - | - |
| | | (0.792-1.128) | (138.670-213.378) | (27.727-32.423) | (45.815-58.636) | | |
| | Viper | 0.024 | 0.96 | 0.013 | 0.22 | - | - |
| | | (0.0196-0.0280) | (0.792-1.128) | (0.0119-0.0142) | (0.195-0.249) | | |

Generally, *D. longispina* was usually significantly affected at lower concentrations of Mikado (AFR: LOEC = 40.0 mg a.i. $L^{-1}$, fecundity: LOEC = 10.0 mg a.i. $L^{-1}$, *r*: LOEC = 20.0 mg a.i. $L^{-1}$, SGR: LOEC ≤ 2.5) in comparison to *D. magna* (AFR: LOEC = 130.0 mg a.i. $L^{-1}$, fecundity: LOEC = 17.5 mg a.i. $L^{-1}$, *r*: LOEC = 130.0 mg a.i. $L^{-1}$, SGR: LOEC = 17.5 mg a.i. $L^{-1}$). However, the fecundity of *D. longispina* showed similar sensitivity to that of *D. magna*, since the 21-d $EC_{50}$s were quite similar (*D. longispina*: 21-d $EC_{50}$ = 52.1 mg a.i. $L^{-1}$, *D. magna*: 21-d $EC_{50}$ = 53.8 mg a.i. $L^{-1}$) (table III.3).

The chronic toxicity of Viper is considerably high either for the autochthonous *D. longispina* or the standard *D. magna*, being SGR the most affected endpoint to which they presented similar LOECs (0.10 and 0.11 mg a.i. $L^{-1}$, respectively) (table III.2). Fecundity and *r* life-history traits showed equivalent trends of significance, though the point estimates computed for *D. longispina* assumed slightly lower values (LOECs = 0.016 mg a.i. $L^{-1}$, 21-d $EC_{50}$ = 0.028 mg a.i. $L^{-1}$) comparatively to *D. magna* (LOECs = 0.052 mg a.i. $L^{-1}$, 21-d $EC_{50}$ = 0.093 mg a.i. $L^{-1}$) (tables III.2, III.3). Likewise, the AFR of *D. longispina* (LOEC = 0.026 mg a.i. $L^{-1}$) was significantly delayed by lower concentrations of Viper than that of *D. magna* (LOEC = 0.088 mg a.i. $L^{-1}$). It should be noticed, however, that data analysis for *D. magna* traits at the highest concentration was constrained by the high mortality rates (see above), and hence by the reduced number of replicates used.

### 3.3.2 Mixture toxicity

Figure III.3 represents the experimental data for mixture toxicity and also the predicted concentration-response curves determined by CA and IA reference models, based on the

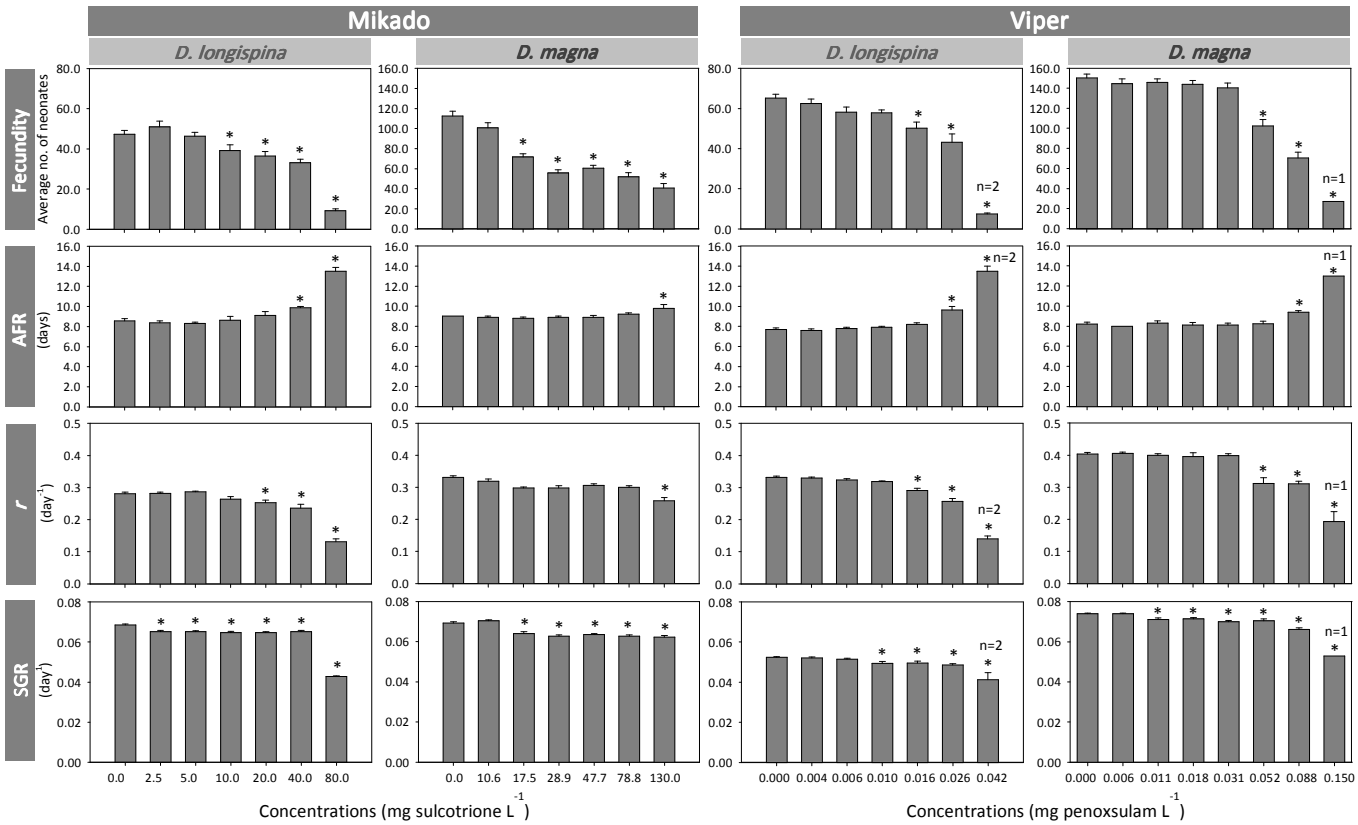


Figure III.2 - Fecundity, age at first reproduction (AFR), intrinsic rate of population increase (*r*) and somatic growth rate (SGR) of *D. longispina* and *D. magna* exposed to several concentrations of Mikado and Viper. Error bars represent standard deviation. Significant differences from the control are signed as * ($P \leq 0.05$).

individual toxicity of mixture components, following application of the log-logistic function. The outcome of model parameters is shown in table III.4.

For *P. subcapitata* growth (expressed as % cell density of the control) both models CA and IA retrieved a significant fit to the observed mixture toxicity data. When testing for deviations from the additive CA model, a better fit was significantly described by a more than additive interaction between mixture compounds, given by the negative value of the model parameter *a*, what indicates synergism to all mixture concentrations (table III.1). The fit of CA model was further improved when it was extended with parameter *b*, hence indicating a significant dose level dependent deviation (DL) from experimental toxicity data. The positive value of parameter *a* revealed an antagonistic effect at lower mixture doses and a synergistic effect at higher doses, occurring the switching between these trends at lower concentrations than the $EC_{50}$ level (*c.f.*, table III.1). Predicted deviation patterns from the IA model were statistically similar to the ones determined for CA. As such, a significant synergistic interaction between mixture compounds was detected to all concentrations, although the most significant deviation pattern from the IA was DL, with antagonism at lower mixture concentrations and synergism at higher ones. The switching

from antagonism to synergism occurred at lower concentrations than the $EC_{50}$ level (*c.f.*, table III.1).

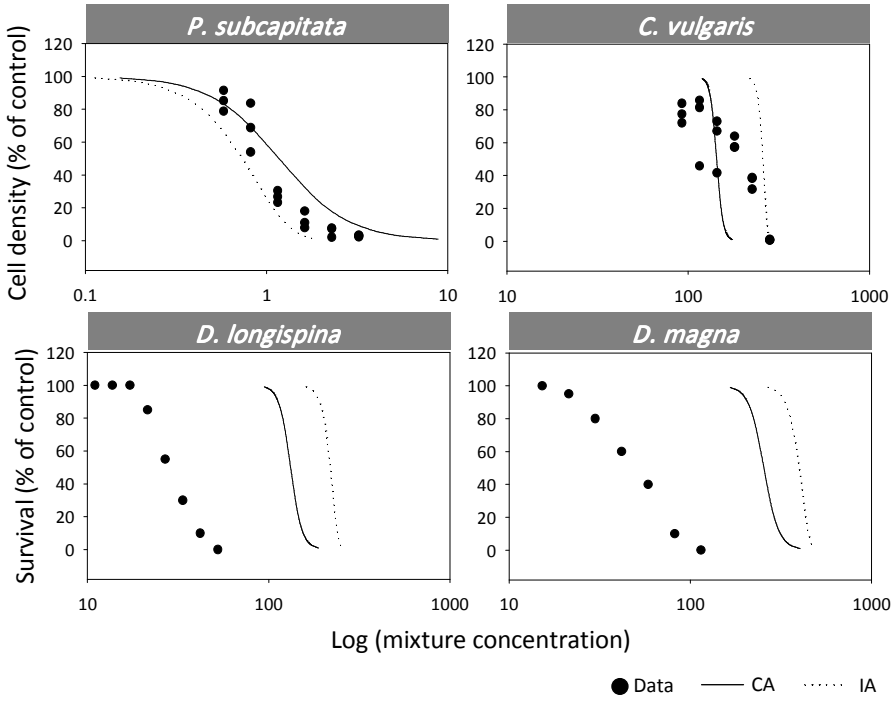


Figure III.3 - CA (straight curve) and IA (dotted curve) predicted curves and data points (black dots) obtained for the mixture effect of Mikado/Viper on algae (*P. subcapitata* and *C. vulgaris*) growth rate and the survival of daphnids (*D. longispina* and *D. magna*).

The mixture toxicity estimated for *C. vulgaris* growth (expressed as % cell density of the control) by CA provided a relative good fit to the observed data (table III.4). However, the positive value of parameter *a* added to the CA reference model indicated a significant antagonistic deviation from it (table III.1). The application of IA concept to the experimental data retrieved a significant fit, but there was a significant synergistic mixture effect estimated by the S/A deviation.

The effect of mixture toxicity on *D. longispina* immobilisation was significantly predicted by the CA concept (table III.4) for the tested concentration range. In spite of this, a better fit was achieved when testing the deviation S/A, indicating a significant synergistic interaction among mixture components to all concentrations. A DL dependent deviation produced even better data fit, characterised by antagonism at lower mixture concentrations and synergism at higher ones, being the switch from antagonism to synergism at lower levels than the $EC_{50}$ (table III.1).

Table III.4 - Outcome summary of mixture effects on algae (*P. subcapitata* and *C. vulgaris*) growth and on the immobilisation of daphnids (*D. longispina* and *D. magna*).

| Species | Model / Deviation type | CA | | | | | IA | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *SS/L* | $r^2$ | *P* | a | b | *SS/L* | $r^2$ | *P* | a | b |
| *P. subcapitata* | Reference | 7581.13 | 0.924 | <0.001 | - | - | 9718.27 | 0.902 | <0.001 | - | - |
| | S/A | 7040.92 | 0.929 | 0.035 | -0.68 | - | 7039.13 | 0.929 | <0.001 | -2.94 | - |
| | DL | 6094.13 | 0.934 | 0.0014 | 2.72 | 1.38 | 6138.19 | 0.938 | <0.001 | 0.47 | 19.22 |
| *C. vulgaris* | Reference | 9138.11 | 0.869 | <0.001 | - | - | 7900.38 | 0.887 | <0.001 | - | - |
| | S/A | 7284.26 | 0.896 | 0.00053 | 1.14 | - | 7217.27 | 0.897 | 0.028 | -1.38 | - |
| *D. longispina* | Reference | 97.76 | 0.727 | <0.001 | - | - | 77.34 | 0.764 | <0.001 | - | - |
| | S/A | 75.48 | 0.789 | <0.001 | -4.45 | - | 72.76 | 0.778 | 0.033 | -2.41 | - |
| | DL | 26.17 | 0.927 | <0.001 | 15.78 | 2.41 | 18.82 | 0.943 | <0.001 | 23.78 | 4.20 |
| *D. magna* | Reference | 167.30 | 0.426 | <0.001 | - | - | 165.91 | 0.430 | <0.001 | - | - |
| | S/A | 18.09 | 0.938 | <0.001 | -6.26 | - | 47.46 | 0.837 | <0.001 | -9.76 | - |

Reference – reference models concentration addition (CA) and independent action (IA); S/A – synergism/antagonism deviation; DL – dose-level deviation; *SS* or *L* –objective functions for continuous and binary data, respectively; $r^2$ – coefficient of determination; *P* – probability of the likelihood ratio test ($\chi^2$) performed between the reference model estimations and the deviation function type; *a* and *b* – parameters of the deviation functions.

IA had also provided a valid adjustment to experimental data, albeit a synergistic deviation from IA was significantly calculated given the negative value of the parameter *a*. Nevertheless, extending the IA with parameter *b* an improved significant fit was obtained by DL deviation, according to which an antagonistic effect occurred at lower concentrations, while at higher concentrations the mixture toxicity had increased (synergism). The shift from antagonism to synergism occurred at a lower dose level than the $EC_{50}$ (table III.1).

Both CA and IA models provided a significant estimate of *D. magna* immobilisation to the tested mixture concentrations (table III.4), although the values of $r^2$ were quite reduced evidencing a poor fit of the models to the observed data. Applying the S/A deviation to CA model, a synergistic effect of mixture was significantly denoted. On the other hand, deviations from IA had also improved its fit to data, thereby indicating a significant synergistic interaction between mixture compounds for S/A deviation type.

## 3.4 Discussion

### 3.4.1 Single compound toxicity

This study provides new ecotoxicological data for the herbicidal formulations of Mikado and Viper on different algae and daphnid species.

Both Mikado and Viper exposures caused impairments on algae growth, and on immobilisation and life-history traits of daphnids, when individually tested. Across the species,

trophic levels and endpoints tested, Mikado was noticeably less toxic than Viper. Apparently, the tested concentrations are slightly (for Viper, *i.e.* penoxsulam) to much (for Mikado, *i.e.* sulcotriona) higher than those found in natural aquatic systems [0.010 - 0.020 µg $L^{-1}$ for sulcotrione (Freitas et al. 2004) and 0.096 – 2.3 µg $L^{-1}$ for penoxsulam (unpublished data)]. However, due to the reduced number of studies available it is not reliable to take solid conclusions on the ecological relevance of the obtained toxicity thresholds. Furthermore, along an agricultural season peak concentrations of herbicides are expected to occur, given the intermittent pattern of pesticide applications (Relyea and Hoverman 2006). Such peak concentrations of herbicides can affect sub-lethal endpoints associated with physiological and/or biochemical changes at the regular metabolism of organisms, hence constraining the structure and function of natural populations and communities (Hanazato 2001).

*a. Microalgae growth assay*

Following exposures to Mikado, *C. vulgaris* presented toxicity values (*i.e.*, LOEC and 96-h $EC_{50}$, tables III.2, III.3) that were two orders of magnitude higher than those of *P. subcapitata*. A similar 96-h $EC_{50}$ for *P. subcapitata* to the one determined in this study was documented by Tomlin (2000) for sulcotrione (1.2 mg a.i. $L^{-1}$), but higher $EC_{50}$s were presented in the AFSSA (2002) database to an unspecified algal species (3.5 mg a.i. $L^{-1}$), and by Bayer CropScience (2004) to *Desmodesmus subspicatus* (10 mg a.i. $L^{-1}$). Notwithstanding, the toxicity of Mikado to the tested algae was seemingly not much higher than that of the a.i. sulcotrione, contrary to what was observed by Bonnet et al. (2008) for two microorganisms. They concluded that the adjuvants added to the formulation of Mikado were probably major contributors to the recorded acute toxicity. Either in microorganisms or algae, the a.i. of Mikado inhibits the enzyme HPPD involved in the tyrosine catabolism to produce plastoquinones and α-tocopherol in chloroplasts. Plastoquinones are fulcral cofactors of phytoene desaturase within the biosynthesis chain of carotenoids, and they are important carriers of the chloroplastic electron-transfer chain at the photosystem II (PSII) (Abendroth et al. 2006, Matringe et al. 2005, Shaner 2003). Concomitantly, the decrease of α-tocopherol pool prevents the quench of singlet oxygen (a reactive oxygen species) (Trebst et al. 2002), thereby leading to the degradation of D1 protein within PSII. All mentioned impairments will then induce a rapid disruption of carotenoid synthesis, destruction of chlorophyll molecules, lipid peroxidation and membrane breakdown ending up with the death of organisms (Abendroth et al. 2006), which is consistent with the observed responses in the present study.

Viper was also remarkably less toxic to *C. vulgaris* than to *P. subcapitata*, while for the cyanobacteria *Anabaena flos-aquae* the FOOTPRINT database (2008) presents an intermediate

penoxsulam toxicity (acute-$EC_{50}$ = 0.27 mg a.i. $L^{-1}$), though the exposure period was not specified. Similar works assessing the toxicity of other sulfonamides (flumetsulam) on *C. vulgaris* observed lower toxicity ($EC_{50}$ = 10.68 mg a.i. $L^{-1}$) (Ma et al. 2002). Whereas sulfonylureas, which present identical m.o.a. and structure-activity relationships to the sulfonamides (Yang et al. 1999), when tested on different green microalgae, including the ones used in this study, showed either higher or lower toxicity ranges (Nyström et al. 1999, Junghans et al. 2003, Ma et al. 2002, Faust et al. 2003, Cedergreen et al. 2007). As such, the toxicity of related xenobiotics is species- and chemical-specific. Thus, it hampers drawing further conclusions except that Viper clearly provoked strong impairments on microalgae growth rates at considerably low concentrations.

Besides the direct effect induced by the a.i. of Viper on algae, the presence of adjuvants in the formulation, such as methanol, may constrain the obtained response pattern. Theodoridou et al. (2002) observed that under low methanol concentrations (up to 0.5%) the biomass of *Scenedesmus obliquus* was enhanced, whilst this parameter was substantially reduced with increasing methanol concentrations, being pointed out its influence on the structure and functioning of photosynthetic apparatus. Therefore, there is a chance that methanol is complementing and enhancing the adverse effect of the Viper a.i. on algae growth.

The m.o.a. of penoxsulam involves the inhibition of the ALS enzyme, what precludes the biosynthesis of essential aminoacids for microalgae growth, though the metabolic pathways responsible for growth impairment were not unravelled yet. Nonetheless, it was advanced that among the possible secondary effects following ALS inhibition, changes on photosynthesis electron transport were likely to occur (Zhou et al. 2007). Under field conditions, however, not only penoxsulam concentrations reaching the aquatic environment may be low (see above), but also, the ability of algae to uptake dissolved aminoacids may help to withstand their growth rates, depending on the period and dose of exposure (Nyström et al. 1999). Källqvist and Romstad (1994) had also indicated that there is a high interspecies sensitivity variation, especially when pesticides with specific m.o.a.s are being evaluated. Indeed, some authors have already reported the lower sensitivity of *C. vulgaris* with respect to some pesticides, comparatively to other green microalgae, namely *P. subcapitata* (*e.g.*, Sabater and Carrasco 1998), corroborating the sensitivity difference herein observed.

*b. Acute and chronic assays with daphnids*

The autochthonous species *D. longispina* was equally or more sensitive than the standard cladoceran *D. magna*, either in acute and chronic assessments, for both formulated herbicides. Antunes et al. (2004) and Pereira and Gonçalves (2007) verified the same sensitivity trend between those species under exposure to other pesticides. Overall and reinforcing what was

above said for algae species, under acute and chronic exposures of daphnids, Viper was also substantially more toxic than Mikado (fig. III.2, tables III.2, III.3).

Comparing the calculated 48-h $EC_{50}$ values for *D. magna* exposed to Mikado with that indicated by Bayer CropScience (2004) for sulcotrione (750 mg a.i. $L^{-1}$), it can be observed that the formulated herbicide was slightly more toxic than the a.i., though they were within the same toxicity range. A similar toxicity pattern was verified for *D. magna* exposed to Viper, given the higher $EC_{50}$ value of penoxsulam (98.3 mg a.i. $L^{-1}$) presented by Dow AgroSciences (2006). Contrary to what was observed for microalgae, both formulated herbicides were apparently more harmful to the survival of *D. magna* than the corresponding a.i.s., as supported by the published information. This result could be indicative of enhanced toxicity addressed by adjuvants added to the formulation (Cox and Surgan 2006), though they are not fully discriminated in their respective data sheets.

It should be still stressed that Viper was deleterious at the µg $L^{-1}$ and low mg $L^{-1}$ levels for the autochthonous and standard daphnid species, respectively, what was unexpected since the known target of action and the corresponding biosynthetic pathway inhibited by Viper a.i. does not exist in animals (*e.g.*, Roberts et al. 2003). However, the presence of methanol in formulation composition (quantities unknown) may induce daphnids' immobilisation. van Wezel et al. (1997) mentioned that methanol may be responsible for neurotoxic effects, which are thought to be due to direct physico-chemical action affecting membrane fluidity. Moreover, it has been reported that short-chain *n*-alkanols like methanol may potentiate the inhibitory activity of gamma amino butyric acid (GABA) receptors (a ligand-gated ion channel governing $Cl^-$ flux), which in turn down-regulates, *i.e.*, inhibits, a wide range of neural transmitter pathways across the nervous system (Zuo et al. 2001), namely those underlying the control of movement in crustaceans (Cattaert et al. 2002). Hence, the locomotor activity of daphnids exposed to Viper may have been constrained to a level that provoked its death.

Though acute tests constitute relevant measures seemingly related with the high pesticide inputs during application pulses, their ecological relevance may be sometimes limited. It is necessary to assess the sub-lethal effects of pesticides at individual-level responses to get a closer overview of potential changes hindered by natural populations (Hanazato 2001) exposed to low concentrations of pesticides usually found in natural aquatic systems.

When *D. longispina* and *D. magna* were subjected to lower concentrations of Mikado, the most affected endpoints were fecundity and SGR, given the obtained lower values of the respective point estimates (tables III.2, III.3). In turn, AFR and *r* were less responsive due to their higher LOEC values (table III.2). FOOTPRINT (2008) database presents a 21-d $EC_{50}$ for *D. magna* under sulcotrione (75 mg a.i. $L^{-1}$) slightly higher than the one obtained in this study for the toxicity

of Mikado on females' reproductive output. The autochthonous species, however, retrieved much lower point estimates, particularly with respect to the population-level endpoint *r*, therefore strengthening its consistently higher sensitivity. Given the lack of studies regarding the biochemical activity of Mikado a.i. on invertebrates, it is not possible to directly ascertain and understand how physiological functions as growth and reproduction are being constrained.

For Viper exposures, SGR was the most affected endpoint for *D. longispina* and *D. magna*, given the lowest and similar LOEC values for both species (table III.2). The endpoints fecundity, *r* and AFR of both daphnid species were impaired under higher Viper concentrations relatively to those determined for SGR, therefore denoting their slightly lower sensitivity to this herbicide. Notwithstanding, the impact of the highest Viper concentrations at the population level was associated to the inhibitory constraints at the individual life-history traits that were integrated by *r* (*i.e.*, fecundity, AFR and mortality). As aforementioned, the target of action of penoxsulam (the a.i. of Viper) does not exist in animals and one possible explanation to the results could be linked to potential inhibitory effects on crustacean movement pre-empted by methanol (an adjuvant of Viper), through a series of neurochemical reactions (see above). As a result, not only the locomotor activity, but also the feeding activities of daphnids may be depressed when exposed to Viper, what subsequently may impair their growth and fecundity, ending up in their mortality.

Indeed, several authors already referred those constraints for daphnids exposed to toxic stress (*e.g.*, Allen et al. 1995, Hanazato 2001, Barata et al. 2006). Although daphnids are able to enhance their fitness through differential energy allocation to guarantee population survival and maintenance, under certain stressful conditions (*e.g.*, starvation or the presence of xenobiotics), physiological functions as growth and reproductive traits may be strongly impaired (Smolders et al. 2005, Pieters and Liess 2006). Consequently, smaller females may produce smaller broods with a delay in the age at first reproduction, thereby triggering the decline in population growth (Allen et al. 1995, Hanazato 2001). This was actually the pattern attained for all the chronic assays herein presented, being further corroborated by significant decreases observed on population growth rates. Although the latter endpoint does not allow direct extrapolations to field scenarios (Forbes et al. 2001), it provides an ecologically reliable measure of pesticide effects on population-level effects while integrating different individual-level effects (Forbes and Calow 1999).

The differential responsiveness of the assessed endpoints strengthens that the m.o.a. of pesticides may elicit different impacts at metabolic and physiological parameters. Assessing the toxicity of such contaminants should thus involve more than one biologically-effect level (*e.g.*, biochemical endpoints). Furthermore, the use of different trophic levels is quite relevant, since it

can provide a better understanding of indirect effects of herbicides on natural communities. This is especially important when dealing, as in this study, with the basis of trophic chains.

### 3.4.2 Mixture toxicity

The current study also provided insight into the combined effects of binary Mikado/Viper mixtures on algae growth and on the immobilisation of daphnids. All data sets were significantly fitted to both CA and IA predictions (*c.f.* table III.4). However, significant deviations from additivity were always detected, thus indicating interactions between Mikado and Viper. Since different results were found for different species and trophic levels, special care is needed for the mixture toxicity evaluation of these chemicals in terms of their interaction and potential extrapolation to other indicator species.

*a. Mixture toxicity on microalgae growth*

Based on the $r^2$ values (table III.4), the strongest predictions retrieved by CA and IA models occurred for algae growth data sets. However, the mixture effect on *P. subcapitata* growth was better described by CA, whereas for *C. vulgaris*, IA was the most accurate predictive model. Yet, a dose level-dependent deviation from CA and IA was significantly determined for *P. subcapitata*, evidencing that at lower mixture concentrations Mikado and Viper interacted antagonistically, while at higher doses their interaction induced synergistic effects. This outcome strengthens the need for higher number of testing combinations and mixture ratios, as a way to cover the response surface as best as possible (Jonker et al. 2005), thereby limiting inconsistent model predictions, as the one observed for IA curve *vs.* data at the light of statistical results (fig. III.3).

The experiment with *C. vulgaris* showed different deviation trends from the additivity models, for the tested concentration range of Mikado/Viper mixture, what may mislead the biological interpretation of the actual mixture toxicity. Relatively to CA, the herbicides interacted antagonistically, while a significant synergistic effect was found for *C. vulgaris* growth against the IA model, thereby denoting that the effect of Mikado was not independent from that of Viper.

The available studies presenting the quantification of these pesticides on aquatic system (in terms of their a.i.s) are mostly absent (see above). However, if lower concentrations of both pesticides are likely to occur on environmental mixtures, it is possible that antagonistic effects on *P. subcapitata* growth and antagonistic/synergistic (according to CA/IA predictions) effects on *C. vulgaris* growth may occur under real exposure scenarios of Mikado and Viper mixtures, considering the effect level and mixture ratio herein tested. Other studies conducted with green microalgae, macrophytes and plants observed the predominant occurrence of antagonistic effects

upon exposure to mixtures containing sulfonylurea herbicides [(other group of ALS inhibitors similar to the sulphonamide group of the a.i. of Viper – penoxsulam (Yang et al. 1999)] (Junghans et al. 2003, Munkegaard et al. 2008) or a binary combination of sulfonylurea and triketone herbicides (mesotrione, an HPPD inhibitor like sulcotrione - the a.i. of Mikado) (Cedergreen et al. 2007).

Different studies showed the predictability power of IA regarding joint effects of dissimilarly acting chemicals on algae (*e.g.*, Faust et al. 2003, Cedergreen et al. 2008). In spite of this, CA had already provided reliable estimates of mixture toxicity on algae, either composed by similar (*e.g.*, Junghans et al. 2003) or dissimilar compounds (*e.g.*, Faust et al. 2003, Cedergreen et al. 2007, Munkegaard et al. 2008). As a matter of fact, the a.i.s of Mikado and Viper present different m.o.a., what *a priori* would lead to the selection of the IA model, but there is a lack of information sustaining an overall understanding of their complete physiological and biochemical pathways across different groups of organisms. However, it was suggested that both a.i.s promote indirect impairments on photosynthesis (*e.g.*, Matringe et al. 2005, Zhou et al. 2007), and that sulcotrione may induce cell membrane destruction (Abendroth et al. 2006). At lower mixture concentrations it is likely that a reduced number of sulcotrione molecules impairing membrane integrity may also reduce the transport of the other chemical [as observed by Syberg et al. (2008) for other pesticides], and somehow mixture effects may be antagonistic or additive. However, under higher mixture concentrations, the action of sulcotrione may strongly enhance the ecotoxicological effect of the other, thereby showing potential synergistic effects. These biochemical changes could explain the dose level-dependent deviation from CA and IA verified for *P. subcapitata*, but no solid conclusions can be draw for *C. vulgaris* response.

*b. Mixture toxicity on the immobilisation of Daphnia sp.*

Although CA and IA showed a significant fit for daphnid immobilisation data, the reduced $r^2$ values (table III.4) indicated that there was a discrepancy between predicted and measured combined effects, especially for *D. magna* response. Overall, both models estimated lower mixture toxicity than that observed experimentally, being CA the model providing the most conservative estimation, similarly to the tendency observed for other mixture studies with cladocerans, involving similar and/or dissimilar pesticides (*e.g.*, Banks et al. 2005, Barata et al. 2006, Cedergreen et al. 2006, Cedergreen et al. 2008, Syberg et al. 2008). Indeed, it has been documented that for endpoints which result from the integration of a complex system of joint actions, such as death and growth, IA is not expected to have higher predictive ability than CA (Faust et al. 2003, Cedergreen et al. 2008). While for *D. longispina* a significant dose level-dependent deviation from the additivity models indicated antagonism at low mixture doses and

synergism at high mixture doses, for *D. magna* was detected a significant synergistic deviation from both CA and IA.

Synergistic effects have been regarded as a great concern under a risk assessment perspective, because very low chemical concentrations may represent a potential hazard when combined (*e.g.*, Cedergreen and Streibig 2005, Cedergreen et al. 2006). Hence, depending on the environmental exposure conditions, Mikado and Viper combination may lead to synergistic sub-lethal effects at sub-individual levels, which may compromise the overall population integrity. Furthermore, it should not be dismissed the possibility that adjuvants added to herbicide formulations enhanced the toxicity of the a.i.s *per se* (as was potentially the case for methanol in Viper formulation, according to what was above explained), thereby enhancing joint synergistic effects of the formulated herbicides as already reported in other studies (*e.g.*, Cabanne and Gaudry 1996). Some published works had also identified synergistic joint effects on the survival of daphnids (*C. dubia* and *D. magna*) for fungicide/insecticide and fungicide/herbicide mixtures (Cedergreen et al. 2006), and for mixtures only composed by herbicides (Banks et al. 2005). It is often explained that the effect of one chemical could be synergised by the presence of the other, due to uptake increase, enhanced activity or inhibition of inactivation both often regulated by changes at the detoxifying/biotransformation processes (*e.g.*, Cedergreen and Streibig 2005). Depending on the duration and magnitude of exposure, such changes may pre-empt complex changes at different physiological/metabolic levels, which may increase energetic cost of detoxification or alter resource allocation patterns supporting the growth and reproduction of individuals, and ultimately lead to their death. However, understanding the whole physiological mechanisms behind chemical joint actions is almost impracticable, at least for the majority of chemicals, even when one is dealing with pesticides having a specific m.o.a. (Barata et al. 2006, Syberg et al. 2008). It could be argued that animals are not the target of herbicides. However, these chemicals may represent important and hazardous contaminants for ecosystems' integrity (Shaner 2003), as far as they affect individual life traits of organisms, as was suggested by the outcome achieved in this study.

Overall, the question about what model should be selected could not be directly answered, as far as both gave valid predictions. However, it has been defended that CA may generally provide the best fit to almost all chemicals (Faust et al. 2003). Under a risk assessment point of view, this is the model that is widely recommended given its usual more conservative estimations of mixture toxicity (Cedergreen et al. 2008, Syberg et al. 2008). In the present study, CA had generally retrieved a significant worst-case prediction relatively to IA, either because the mixture effects were overestimated (antagonism detected for both algae species growth) or less sub-estimated (for both daphnid species immobilisation) than IA.

In a general view, to attain more consistent conclusions about mixture joint effects of these two herbicides, not only pharmacological studies across different species and trophic levels are needed, but also other endpoints should be assessed. As strengthened by Cedergreen and Streibig (2005), the choice of endpoints may provide different conclusions and predictions of mixture toxicity given by CA and IA models, thus, their selection should reflect, as better as possible, the m.o.a. of the chemical. As such, future work may also include sub-lethal biochemical (*e.g.*, enzyme activities at different metabolic pathways) and other individual-level (*e.g.*, feeding rates together with different life-history traits of daphnids) endpoints to assess different mixture effect levels, mixture ratio and concentration ranges. Moreover, such experimental design should further be applied to other ecologically relevant trophic levels and species, in order to achieve a more comprehensive understanding about Mikado and Viper combined action under potential environmental aggression scenarios.

## 3.5 Conclusions

The present study generated individual and combined ecotoxicological data of Mikado and Viper on two trophic levels that, according to authors' knowledge, had not been published so far. On a whole, the assessment of single-compound effects on algae growth and on the acute immobilisation and chronic life-history traits of daphnids evidenced that Viper was generally one to three orders of magnitude more toxic than Mikado. In most cases, the lowest point estimate values indicating higher toxicity were calculated under sub-chronic (for algae) and chronic (for daphnids) exposures, what reinforces that the evaluation of sensitive sub-lethal endpoints should be addressed in such-like studies, since sub-lethal effects are more likely to occur under field conditions than acute effects. Additionally, it was pointed out that the toxicity of both formulated herbicides could have been enhanced due to the presence of adjuvants, especially for Viper. Notwithstanding, further studies are needed towards the assessment of those herbicides at lower biological-level endpoints that could be more closely related with their m.o.a. or the metabolism of organisms.

Although CA and IA provided an accurate description of Mikado and Viper joint action on algae growth and immobilisation of daphnids, significant deviations from additivity were always detected. A low-dose antagonism and high-dose synergism was observed from both models for *P. subcapitata*, whereas *C. vulgaris* response deviated antagonistically from CA and synergistically from IA. For the immobilisation of both daphnids, however, synergistic effects were observed for higher Mikado/Viper mixture concentrations, from both models. Thereby, the decision of what model provides the best prediction is not straightforward, since when complex endpoints and

organisms are assessed an array of toxicodynamic and toxicokinetic interactions may co-occur hence misleading the CA and IA concepts and turning impracticable the classification of compounds based on one single m.o.a. Under a regulatory standpoint, the model to choose should provide the most conservative estimation, which in this study was the CA model either because the mixture effects were overestimated or less sub-estimated than IA. Nevertheless, it is suggested that further pharmacological and ecotoxicological studies, using more comprehensive mixture experimental designs, different physico-chemical stressors, sub-individual sensitive endpoints and trophic levels should be conducted to accomplish a more reliable overview of Mikado/Viper mixture effects.

Overall, the great sensitivity differences observed within species did not allow the conclusion that one trophic level was more tolerant than the other. Instead, *P. subcapitata* was always the most sensitive species to both herbicide formulations, followed by *D. longispina*, whilst *D. magna* and *C. vulgaris* were the most tolerant species.

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# Chapter IV

Using earthworm avoidance behaviour to assess the toxicity of formulated herbicides and their active ingredients on natural soils

# Using earthworm avoidance behaviour to assess the toxicity of formulated herbicides and their active ingredients on natural soils

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## Abstract

This work aims to assess the effects of two herbicide active ingredients (a.i.) — sulcotrione and penoxsulam—and their respective commercial formulations—MIKADO® and VIPER® (referred as Mikado and Viper)—on the avoidance behaviour of *Eisenia andrei*. The avoidance tests were run with standard (LUFA 2.2; L) and natural soils (from corn and rice fields), as long as their habitat function did not constrain the earthworm behaviour. The physico-chemical characterisation of soils was also performed. The avoidance tests intended to ascertain (i) the random distribution of earthworms in the natural soils C and R (dual-control tests), (ii) the habitat function of natural soils against each other and against L soil, (iii) the effect of a.i.s and formulated herbicides on *E. andrei* behaviour. Avoidance tests with the a.i.s were only performed in L soil. C and R soils presented higher organic matter (OM) and clay/silt contents and water holding capacity than L soil. Earthworms distributed randomly in dual-control tests, but preferred R soil significantly, relative to L or C soils. The response of earthworms could be related to the quantity OM content and quality of organic and inorganic fractions of soil, beyond other intrinsic properties of soils. The behaviour of *E. andrei* was more affected under penoxsulam or Viper exposures on L soil, being the latter formulated product even more repellent for *E. andrei* than the a.i. Hence, the effect of adjuvants on Viper may have increased the toxicity of the a.i. Our results reinforced the need for a careful assessment of the impacts of formulated products. Furthermore, since there was a reduction in earthworm % avoidance under Viper exposures on the natural soil R, it was possible that pesticide bioavailability had been reduced by its sorption to OM and clay mineral sorption sites. In conclusion, though the standard L soil should be used for reproducibility and comparison means, other natural soils should be added to the assessment of chemicals, for sake of ecological relevance. Overall, avoidance tests provided a sensitive, valuable and feasible response either to compare the habitat function of different standard and agricultural natural soils or to test the effect of herbicides.

**Key-words:** behavioural endpoint, *Eisenia andrei*, herbicides, Mikado®, Viper®, natural soils.

## 4.1 Introduction

Recent awareness regarding the urgent need for soil protection (CEC 2006) encouraged the development of frameworks for the prospective risk assessment (EC 2003) of new and existing chemicals, as well as pesticides (EEC 1991, EC 2002). Such an assessment approach suggests the performance of tests with earthworms to study the toxicity of pesticides upon the use of standard acute and chronic tests (EEC 1991, EC 2002, EC 2003).

Indeed, a wealth of literature points out for earthworms, as a key ecological receptor widely used in ecotoxicological studies and that they are also one of the terrestrial organisms potentially exposed to the presence of pesticides in soil (Muthukaruppan et al. 2005, Reinecke et al. 2002, Reinecke and Reinecke 2007, Römbke 2006). This can be attributed, on the one hand, to their ecological role in the maintenance of soil structure and functioning, mainly sustained by their burrowing activities, and to their breakdown of organic matter (Lavelle et al. 2006, Römbke et al. 2005). On the other hand, earthworms are sensitive to the presence of chemicals in the soil due to the chemoreceptors distributed on their body surface (Reinecke et al. 2002). This characteristic associated with their locomotory abilities, renders them the chance to avoid contaminated areas where soil habitat function has been affected (Reinecke et al. 2002, Yeardley et al. 1996).

The avoidance behaviour of earthworms has been defended as an ecologically relevant endpoint (*e.g.*, Hund-Rinke and Wiechering 2001, Amorim et al. 2005) to be used as an indicator of soil quality in a sublethal test—the earthworm avoidance test (ISO 2005). The advantages of avoidance tests rely on their short duration and reduced effort comparative to the acute or chronic tests, being generally more sensitive than the acute tests, while, according to some authors, they respond similarly to the reproduction tests (Achazi 2002, Garcia et al. 2008, Hund-Rinke and Wiechering 2001, Hund-Rinke et al. 2005, Yeardley et al. 1996).

Earthworms have been demonstrated to avoid soils contaminated with pesticides, mainly with fungicides (*e.g.,* Garcia et al. 2008, Natal-da-Luz et al. 2008, Zhou et al. 2007) and insecticides (*e.g.,* Reinecke and Reinecke 2007), but there is not much published information about detrimental impacts triggered by herbicide applications on behavioural endpoints. In spite of this, large quantities of herbicides are used worldwide and, in 2002, they represented ca. 35% of the pesticides used in Europe (ECPA 2003). Although herbicides are not designated for the control of animal pests, the bioavailability of their residues in the soil matrix may threaten the maintenance of earthworm and other soil invertebrates.

On these grounds, the aim of the present study was to assess the effects of two herbicide a.i.s —sulcotrione and penoxsulam—and their respective commercial formulations—MIKADO® and VIPER® (hereinafter referred to as Mikado and Viper)—on the avoidance behaviour of *Eisenia andrei*.

In an attempt to enhance the ecological relevance of the generated toxicity data, the avoidance tests were run with standard (LUFA 2.2; L) and agricultural [from corn (C) and rice (R) fields] natural soils.

This work makes part of a more comprehensive study, concerning an agricultural area intensively exploited for corn and, especially, rice production, in which Mikado and Viper are applied, respectively. They are relatively new herbicides on the European market (Meazza et al. 2002, Bird et al. 2006) and the related available ecotoxicological studies are scarce, as far as authors are aware. Although the registration process complies with the evaluation of the a.i. and the 'lead formulation' (EEC 1991), the available ecotoxicological data of the commercialised pesticides relies mainly on the acute effects induced by the a.i.s. According to Tominack (2000) and Cox and Surgan (2006), the toxicity of adjuvants added to pesticide formulations is often more toxic to non-target living organisms than the a.i.; what strengthens these formulations should be carefully assessed and the data communicated. Therefore, it is quite noteworthy to compare and produce toxicity data based on rapid sublethal endpoints that could additionally provide a more ecologically-sound outcome of potential damages on non-target organisms. On the other hand, the present work will contribute to enlarge the terrestrial ecotoxicological database, which is considerably poor and needs urgent updates for the derivation of soil quality thresholds that are useful for the protection of terrestrial ecological receptors (O'Halloran 2006).

## 4.2 Material & Methods

### 4.2.1 Test organisms

The epigeic earthworm *E. andrei* (Lumbricidae) was bred in large plastic boxes containing a mixture of horse manure, dried leaves and potting soil as substrate, which was regularly moistened and monitored for pH levels. The culture was maintained at temperature 20 $\pm$ 2ºC and photoperiod $16^{L}$:$8^{D}$ h. One day prior to the beginning of the test, adult worms presenting developed clitella with an average weight of 300 – 600 mg, were selected and kept in the pre-moistened standard soil L for acclimatisation.

### 4.2.2 Soils

In the present study, two natural soils and one standard natural soil (hereinafter referred to as standard soil) were used. The natural soils were collected in the 0 – 20 cm soil surface layer from corn (C) and rice (R) fields, before the cropping season to guarantee that there was no recent input of pesticides. These fields are integrated in a wide area extensively used for agriculture in the Lower Mondego river Valley, which is located in the centre of Portugal (40º 2' N, 8º 43' W). In the

laboratory, both soils were air-dried, homogenised and sieved (2-mm mesh) before their characterisation and the performance of avoidance tests.

The standard soil used was LUFA 2.2 (commercially available at Agricultural Research Centre, Speyer, Germany). In temperate regions, this European soil is widely accepted as a suitable and reference soil for conducting ecotoxicological assays with invertebrates (Løkke and van Gestel 1998), namely avoidance tests (Garcia et al. 2008). The physico-chemical characterisation of L soil shown in table IV.1 was provided by Agricultural Research Centre, Speyer, Germany.

Relativly to the characterisation of natural soils, ten replicates were used to measure the pH ($H_2O$) (FAOUN 1984), pH (KCl) (ISO 2005), conductivity (FAOUN 1984) and organic matter content (OM) (SPAC 2000). The pH ($H_2O$ or KCl) and the conductivity were determined in a soil suspension of 1:5 (w/v) soil:water (or KCl 1M). After 30 minutes of shaking thoroughly, the suspension was left to rest for 1 h before measuring the pH of the overlying solution with a WTW 330/SET pH meter. On the day after, the conductivity was recorded with the WTW LF/330 meter. The OM content of each replicate was obtained by ignition loss at 450ºC during 8 h. The maximum water-holding capacity (WHC) (ISO 2005) was determined in three replicates for each soil type. Soil samples were introduced in plastic vessels and immersed in tap water for 3 h. Afterwards, they were drained for 2 h, weighed, dried at 105ºC until the weight was stabilised, and re-weighed again to obtain the WHC. The particle size distribution was determined in one replicate of each soil type (FAOUN 1984). All samples were pre-treated with hydrogen peroxide to destroy OM, and then mixed with a sodium hexametaphosphate solution to enable particle desegregation. The different fractions were separated via mechanical shaking and the use of different pore sieves (2 mm, 1 mm, 500 µm, 250 µm, 125 µm and < 63 µm), although only the clay/silt content (< 63 µm) will be shown. The whole physico-chemical characterisation procedure is further described by Pereira et al. (2008).

### 4.2.3 Chemicals

Mikado, marketed in Europe by Bayer CropScience, is a foliar-applied post-emergence herbicide mostly used in corn crops through terrestrial application, for the control of broadleaf weeds and annual grasses (ter Halle et al. 2006). Mikado is produced as a concentrated suspension containing 300 g a.i. $L^{-1}$, being its recommended rate of application of 1.5 – 2 L $ha^{-1}$. Its a.i. is sulcotrione, a 2-benzoylcyclohexanodione from the triketone class of compounds, whose mode of action relies on the inhibition of the enzyme *p*-hydroxyphenylpyruvate dioxygenase (HPPD). In plants, HPPD is involved in the biosynthesis of plastoquinones and vitamin E. The inhibition of HPPD contributes to the bleaching of plants, due to carotenoid depletion and consequent destabilisation of the photosynthetic apparatus, followed by necrosis and death (Chaabane et al. 2007, Matringe et al. 2005). Sulcotrione presents a water solubility of 165 mg $L^{-1}$ (25ºC) and its degradation rates in soil

vary between 15 – 74 days in the laboratory and 1 – 11 days in the field (Tomlin 2000). Sulcotrione $K_{oc}$ values range between 44 (high pH, sandy clay loam soil type) to 940 (low pH, sandy soil type) (Tomlin 2000). The analytical grade compound (CAS no. 99105-77-8) needed for the avoidance tests with the a.i. was provided by Bayer CropScience, Monheim, Germany. The WHO (World Health Organisation) classified sulcotrione as moderately hazardous (Bayer CropScience 2004).

Viper (Dow AgroSciences) is also a post-emergence herbicide, though it is applied in rice fields via terrestrial or aerial spraying, for the control of annual grasses, sedges, and broadleaf weeds (Roberts et al. 2003). Its formulation type is oil dispersible, containing 97.86% of other ingredients, including an adjuvant that has methanol (information provided by Dow AgroSciences fact sheet). Viper is applied at a rate of 2 – 2.5 L $ha^{-1}$. The a.i. of Viper is penoxsulam ([20.4 g a.i. $L^{-1}$), a triazolopyrimidine sulfonamide compound, which acts as an acetolactate synthase (ALS) inhibitor, targeting the biosynthesis of branch-chained amino acids (valine, leucine, isoleucine), a metabolic pathway found in fungi, microorganisms and plants (Roberts et al. 2003, Jabusch and Tjeerdema 2005). Thereby, ALS inhibition decreases protein and enzyme synthesis, resulting in a rapid cessation of organism growth. The solubility of penoxsulam in water is pH-dependent [0.0057 g $L^{-1}$ at pH 5, 0.41 g $L^{-1}$ at pH 7 and 1.46 g $L^{-1}$ at pH 9 (all at 19°C)], and its $K_{oc}$ is 104 (Roberts et al. 2003). Penoxsulam soil half-life varies between 2 – 118 days, depending on the degradation pathway (U.S. EPA 2007). According to WHO (2005), penoxsulam is unlikely to present acute toxicological hazards under normal use. Analytical standard samples of penoxsulam (CAS no. 219714-96-2) were obtained from Dow AgroSciences LLC.

### 4.2.4 Avoidance tests

Following the procedures established by ISO (2005), the avoidance tests were carried out in two-chamber glass recipients (area = 0.026 $m^2$), which were separated by a card divider, before the introduction of 200 g dry soil into the control (left side) and test (right side) sections (either contaminated with pesticide or not—as in the case of testing a natural soil for its habitat function quality, based on its intrinsic properties). Afterwards, soil water content was adjusted to 40% of the WHC (previously determined as described above) with distilled water for the standard soil L, and to 27 and 28% for R and C soils, respectively. The latter moisten percentages were lower, since the natural soils were too clayed to retain more water without compromising earthworm maintenance. Ten earthworms, previously washed and dried with absorbent paper were then placed in the line dividing the two sections, after withdrawing the card divider. Finally, the recipients were wrapped with a transparent and perforated plastic cover, being left for 48 h under the same conditions as the breeding cultures. After that period, the control and test soils were separated and the number of earthworms in each section was counted as described in ISO (2005). Two validity criteria were

assured for the correct performance of the avoidance tests: i) random distribution of earthworms on both sections of the recipient test when filled with the same uncontaminated soil, ii) no mortality (Hund-Rinke and Wiechering 2001).

*a. Dual-control tests and habitat function of natural soils*

The use of avoidance as an endpoint assumes that earthworms are randomly distributed in the two sections of the testing recipient containing the same soil type (Hund-Rinke and Wiechering 2001, Yeardley et al. 1996). Thereby, in an attempt to validate this criterion, dual-control tests were performed with 10 replicates for each natural soils C and R, testing the same uncontaminated soil type in both sections. Since L is considered a reference soil, it was assumed that earthworms presented a random and homogeneous distribution under such conditions.

Additionally, the habitat function provided by the natural soils coming from the rice and corn fields was tested as well, against the standard soil L (i.e. L vs. C, and L vs. R), and against one another (i.e. R vs. C). Ten replicates were used as well for each test combination. The evaluation of natural soils' habitat function will allow one to ascertain if their pedological characteristics *per se* constrain earthworm's maintenance. Whenever a natural soil was significantly avoided by earthworms, it was not used for the subsequent ecotoxicological assessment of pesticides, as long as the reduced habitat function of the natural soil could mask earthworm behaviour to pesticide effects.

*b. Toxicity of active ingredients and formulated herbicides*

The soils used as substrates for the avoidance assays with pesticides were the standard soil L and the natural soil R, as neither of them was significantly avoided by earthworms (c.f. results' section).

Overall, their spiking was done by thoroughly mixing the test solution with one batch of soil, before introducing it into the test vessel (ISO 2005). Before placing the earthworms in the test vials, the testing solution was led to equilibrate in soil matrix for 1 – 2h. Four replicates were carried out for each tested concentration.

The avoidance tests conducted with the a.i.s were only performed on L soil for the concentrations 100, 250, 500, 750, 1000 mg sulcotrione $kg^{-1}$ and 3, 15, 30, 60, 100 mg penoxsulam $kg^{-1}$. Before the test started, test solutions were individually prepared for each concentration. The respective quantities of sulcotrione were dissolved in 4 mL acetone (99% purity), thereby enabling the contamination of four replicates *per* concentration. A negative control was run in our lab (i.e. an avoidance test with LUFA 2.2 contaminated by 1 mL acetone in the test section per replicate), and it was concluded that the solvent was not constraining the earthworms' response (unpublished data). In turn, each penoxsulam test solution was obtained by dissolving the respective quantity of reagent

in distilled water (at pH 9 and 19ºC) followed by ultrasonic dispersion, before being mixed in a LUFA 2.2 soil batch.

The tested concentrations for each formulated herbicide were defined according to their recommended application rates, corresponding to 3.96 mg a.i. $kg^{-1}$ for Mikado and 0.33 mg a.i. $kg^{-1}$ for Viper. Since no avoidance behaviour was verified at that level, higher nominal concentrations, arranged in a geometric series, were tested: 126.6, 253.2, 506.4, 1012.8, 2025.7 mg a.i. $kg^{-1}$ for Mikado, and 23.4, 35.1, 52.7, 79.0, 118.5 mg a.i. $kg^{-1}$ for Viper, respectively. The test solutions were prepared with distilled water in the same way as aforementioned.

#### 4.2.5 Data analysis

The results of dual-control and soil comparison tests were presented as the average number of earthworms on the test soil per test vessel, for each combination, according to ISO (2005) guidelines. However, a percentage of effect (% avoidance) could be calculated for the testing of chemical contaminants on uncontaminated soils, following the expression: % avoidance = ((E - T) / E) x 100, where E is the expected number of worms in the control soil assuming an homogeneous distribution of earthworms in the test recipient (if N = 10, than E = 5), and T is the average number of worms counted in the test soil per concentration (ISO 2005). Hence, the transformed data could then be used for subsequent statistical analyses, considering negative responses as 0% avoidance.

Notwithstanding, the calculation of an avoidance effect resulting from the testing of chemicals slightly differs in published works, which may cause misunderstandings, *e.g.,* regarding the application of methods for data transformation and its respective interpretation. Some authors (*e.g.,* Amorim et al. 2005, Garcia et al. 2008, Antunes et al. 2008) expressed the avoidance effect of chemical contaminants as the average percentage of net response [i.e. NR = ((C - T) / N) x 100, where C = sum of worms found in the control soil, T = sum of worms found in the test soil, N = total worms per replicate], while Loureiro et al. (2005) calculated avoidance as A = (N – 2 x T) / N, where N = number of worms per replicate and T = number of worms in the test soil. In fact, the final outcome is similar to the one obtained with the equation suggested by the guideline (ISO 2005). However, the mathematical reasoning that sustains the ISO % avoidance expression is more coherent with the expected random migration of earthworms through both test sections, which corresponds to the no-avoidance or no-effect situation that is considered as null-hypothesis when performing statistical comparison tests.

Two main approaches were used for data assessment: (i) application of a threshold value and (ii) statistical analyses. The threshold value-method considers that a test soil presents limited habitat function when > 80% of earthworms are in the control soil (or < 20% are in the test soil) (Hund-Rinke and Wiechering 2001), which corresponds to > 60% avoidance [from the expression suggested by ISO

(2005) for the calculation of % avoidance, if N = 10, then [((5 – 2) / 5) x 100 = 60%]. This evaluation criterion is a less sensitive approach, in comparison with the statistical one, and this is the reason why it was initially proposed to minimise the influence of different physico-chemical properties between the reference and site contaminated soils on earthworm behaviour (Hund-Rinke et al. 2005). Nevertheless, both methods are often used together, as a potential way of improving the robustness of data interpretation (*e.g.,* Sousa et al. 2008).

Therefore, regarding the statistical approach, different analyses were made. First, a pairwise *t*-test was conducted in order to compare the number of earthworms in the control and test sections for the dual-control tests and those intended to compare the quality of different soils. Secondly, for the testing of herbicides, a one-way analysis of variance (one-way ANOVA) followed by the *post hoc* Dunnett's test (Zar 1996) was used to assess significant differences of the % avoidance values between individual chemical concentrations and the control, for each treatment (the control was considered to be 0% avoidance for L soil and equal to the % avoidance-value calculated for dual-control tests carried out with the C and R soils), thereby allowing the determination of NOEC (no-observed effect concentration) and LOEC (low-observed effect concentration) values. Third, and also just for the testing of herbicides, a Probit regression analysis was applied to the % avoidance data in order to determine the effect concentration at a 50% level ($EC_{50}$) and its respective confidence limits at 95% probability (95%-CL) (Finney 1971). As described in the guideline (ISO 2005), the $EC_{50}$ of an avoidance test represents 75% of the earthworms in the control section and 25% in the test soil. If the worms distribute randomly in the test vessel (no-effect situation), at the end of the exposure period there will be 50:50% (if N = 10, it will be 5:5 worms) in each side. However, if there is avoidance behaviour and half of the earthworms (i.e. 50% of the 5 earthworms in the test soil) move from the test soil to the control one, it means that 2.5 or 25% of them avoided staying in the test side, what corresponds to 50% of effect [i.e. ((5 – 2.5) / 5) x 100 = 50%].

## 4.3 Results

The physico-chemical properties of the standard and natural soils used as substrate tests are described in table IV.1. Natural soils C and R presented slightly higher pH ($H_2O$) than L soil, while the latter had the highest conductivity value (57.2 µS $cm^{-1}$). Since both natural soils are characterised as very clayed soils (53.3 and 43.1% clay/silt for C and R soils, respectively), the recorded OM content (5.3 and 4.5% for C and R soils, respectively) and WHC (107.2 and 109.9% for C and R soils, respectively) are more elevated than those in L soil (4.1 and 48.0%, respectively).

Table IV.1 - Physico-chemical characterisation (value ± standard error when available) of the natural standard soil LUFA 2.2 (L) and the natural soils collected in a corn (C) and a rice field (R).

| Soils | pH (KCl) | pH ($H_2O$) | Conductivity (mS $cm^{-1}$) | OM (%) | WHC (%) | Clay/Silt (%) | Sand (%) | Soil texture class[a] |
|---|---|---|---|---|---|---|---|---|
| L | 5.6±0.4 | 5.9±0.1 | 57.2±1.1 | 4.1±0.03 | 48.0±3.0 | 21.4 | 79.1 | Loamy sand |
| C | 5.7±0.02 | 6.8±0.02 | 11.5±0.2 | 5.3±0.2 | 107.2±2.3 | 53.3 | 46.8 | Clay |
| R | 5.4±0.01 | 6.6±0.03 | 15.2±0.2 | 4.5±0.1 | 109.9±12.9 | 43.1 | 50.8 | Clay |

[a]According to the British textural triangle (Gerrard 2000). OM—organic matter, WHC—water holding capacity.

In a general view, both validity criteria were fulfilled for the avoidance tests once no earthworms died and their distribution between the two chambers was approximately 50:50% in the dual-control tests carried out with the natural soils C and R (fig. IV.1). As such, there was no significant avoidance behaviour (table IV.2) when the same uncontaminated natural soil was placed in each side of the test recipient. However, when assessing the natural soils' habitat function, the pairwise *t*-test (*c.f.,* table IV.2) pointed out that earthworms had significantly avoided the C soil when tested against L and R soil (i.e. for L-C and R-C combinations), whilst they preferred R soil relatively to the L one (i.e. for L-R combination) (*c.f.,* fig. IV.1). Since the habitat function of C soil is impacted concerning earthworm maintenance (18.9 and 19.4% earthworms were in the soil C when tested against L and R soils, respectively), it was not considered for further testing of the formulated herbicide applied in corn fields—Mikado—, as it could mislead the interpretation of the response of earthworms.

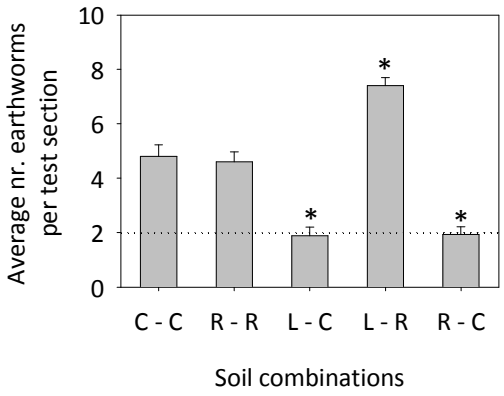


Figure IV.1 - Average number of earthworms in the test soil (the one on the right side of hyphen) for dual-control tests (combinations C-C and R-R) and the comparison of different soils (combinations L-C, L-R and R-C). L (LUFA 2.2), C (corn field soil), R (rice field soil). Error bars represent standard error. Asterisk (*) indicates a significant difference on earthworm distribution between the two sections for each combination, pairwise *t*-test, $P \leq 0.05$.

Table IV.2 - *t*-test (*t*) statistical outcome, regarding the avoidance behaviour of *E. andrei* for soil comparison (L-C: LUFA 2.2 *vs.* corn field soil, L-R: LUFA 2.2 *vs.* rice field soil, R-C: rice field soil *vs.* corn field soil) and dual-control tests (C-C: dual-control test for corn field soil, R-R: dual-control test for rice field soil), and for pesticide exposures.

| Soil comparison and dual-control tests | | | |
|---|---|---|---|
| Tes/Soil combinations | *t* | *d.f.* | *P* |
| L – C | 10.058 | 8 | ≤ 0.001 |
| L – R | – 8.101 | 9 | ≤ 0.001 |
| R – C | 8.182 | 7 | ≤ 0.001 |
| C – C | 0.732 | 9 | 0.483 |
| R – R | 1.078 | 9 | 0.309 |

*d.f.* —degrees of freedom, *P*—probability

Avoidance tests conducted for the testing of herbicides had generally depicted a positive concentration-effect relationship (figs. IV.2, IV.3). In doing so, the LOEC (> 1000 mg a.i. $kg^{-1}$) and $EC_{50}$ (1263.3 mg a.i. $kg^{-1}$) values determined for the behaviour of earthworms when exposed to sulcotrione were slightly lower relative to those calculated for Mikado exposures (1012.8 and 1301.3 mg a.i. $kg^{-1}$, respectively) (table IV.3), using the soil L as substrate. Accordingly, the habitat function limit criterion of 60% avoidance was surpassed under the two highest Mikado concentrations (1012.8 and 2025.7 mg a.i. $kg^{-1}$), whereas the % avoidance for sulcotrione was always below that limit.

Table IV.3 - Summary of the one-way analysis of variance (*F*) for the % avoidance of *E. andrei* exposed to pesticide active ingredients (sulcotrione and penoxsulam) and respective formulations (Mikado and Viper). The NOEC (no-observed effect concentration) and LOEC (low-observed effect concentration) values are also presented, followed by the $EC_{50}$s (concentration that provokes a 50% effect) and respective 95%-confidence limits (CL). L (LUFA 2.2) and R (rice field soil) refer to the used soil types.

| Active Substance/product | Soil type | *F* | *d.f.* | *P* | NOEC | LOEC | $EC_{50}$ | 95% – CL |
|---|---|---|---|---|---|---|---|---|
| | | | | | (mg a.i. $kg^{-1}$) | | | |
| Sulcotrione | L | 2.168 | 5,18 | 0.104 | ≥ 1000 | > 1000 | 1263.3 | ND |
| Penoxsulam | L | 3.674 | 5,18 | 0.018 | 60 | 100 | 80.6 | ND |
| Mikado | L | 9.320 | 7,22 | <0.001 | 506.4 | 1012.8 | 1301.3 | 904.82 – 2170.92 |
| Viper | L | 30.017 | 6,21 | <0.001 | 35.1 | 52.7 | 51.5 | ND |
| | R | 5.499 | 6,21 | 0.001 | 52.7 | 79.0 | 56.9 | 39.37 – 83.59 |

ND—not determined, *d.f.* —degrees of freedom, *P*—probability

Avoidance tests with penoxsulam on L soil resulted in a significant % avoidance under the highest tested concentration (*c.f.*, table IV.3, fig. IV.3a), being the LOEC of 100 mg a.i. $kg^{-1}$ and the $EC_{50}$ of 80.6 mg a.i. $kg^{-1}$, which were higher than those point estimates calculated for Viper (LOEC = 52.7 and $EC_{50}$ = 51.5 mg a.i. $kg^{-1}$), although the $EC_{50}$s were within the same range (*c.f.*, table IV.3). The concentrations of penoxsulam and Viper that had induced significant avoidance response coincided with the ones inducing a limited habitat function of the respective soils, according to Hund-Rinke and Wiechering (2001) criterion. Regarding the testing of Viper in R soil (*c.f.*, fig. IV.3c), the % avoidance was significantly enhanced when earthworms were exposed to the two highest concentrations (*c.f.*, table IV.3, fig. IV.3c; LOEC = 79.0 mg a.i. $kg^{-1}$ and $EC_{50}$ = 56.9 mg a.i. $kg^{-1}$) (*c.f.,* table IV.3), being the habitat function of the test soil impaired (i.e. % avoidance was > 60%) for the same concentrations. Thereby, though within the same range, the toxicity of Viper on L soil was slightly higher than that verified in the avoidance tests conducted with R soil as substrate (*c.f.*, fig. IV.3b vs. IV.3c). This was supported by the lower values calculated for the point estimates in the former set-up—L soil contaminated with Viper (*c.f.,* table IV.3). Overall, earthworms depicted more elevated % avoidance under lower concentrations of penoxsulam or Viper than those of sulcotrione or Mikado (*c.f.*, figs. IV.2, IV.3, table IV.3).

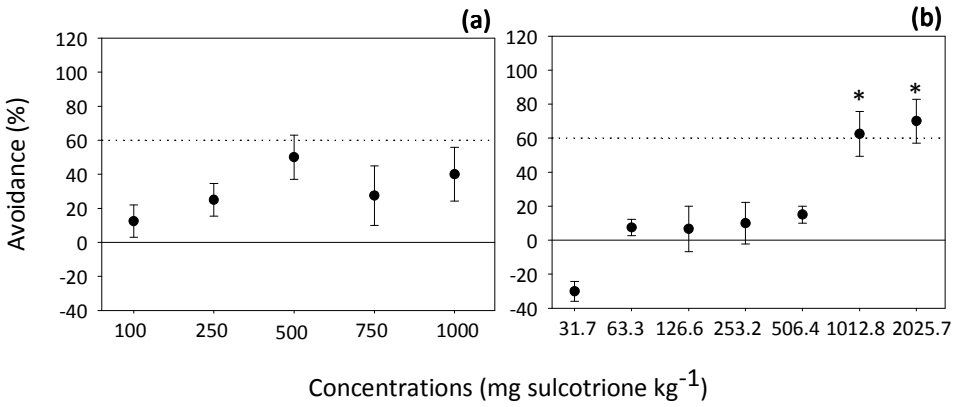


Figure IV.2 - Average percentage of *E. andrei* avoidance response under different concentrations of the (a) active ingredient sulcotrione and the (b) formulated herbicide Mikado, on standard soil LUFA 2.2. Error bars represent standard error. Asterisk (*) indicates a significant avoidance response, one-way ANOVA, $P \leq 0.05$.

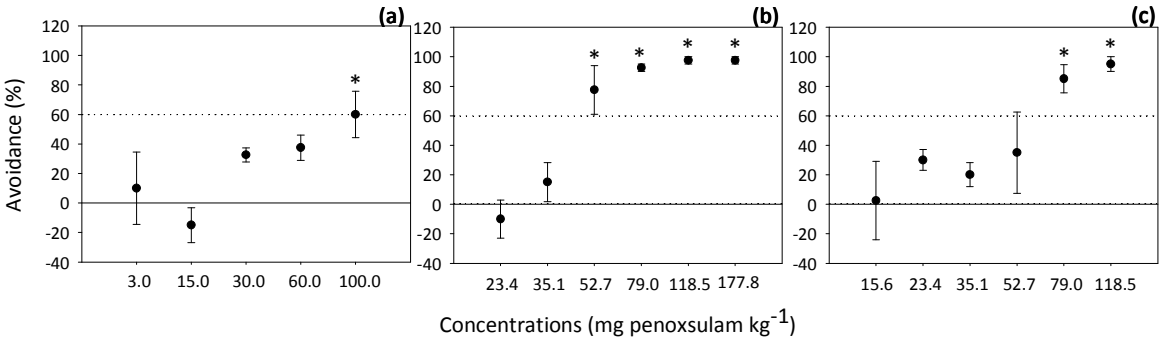


Figure IV.3 - Average percentage of *E. andrei* avoidance response under different concentrations of the (a) active ingredient penoxsulam and the (b) formulated herbicide Viper on LUFA 2.2, and of the (c) formulated herbicide Viper on the natural rice field soil. Error bars represent standard error. Asterisk (*) indicates a significant avoidance response, one-way ANOVA, $P \leq 0.05$.

## 4.4 Discussion

The first part of this study attempted on the evaluation of the role of intrinsic physical and chemical properties of natural soils on their habitat function. In fact, the pedological properties such as texture, pH, and OM content can present a wide range between different natural soils (Jänsch et al. 2005). Therefore, the individual soil properties must be considered when natural soils are used, as well as their suitability as a habitat by earthworms, must be ascertained prior to testing (Edwards and Bohlen 1996).

At the light of the obtained results, the dual-control avoidance tests evidenced a random distribution of organisms either for C or R soils. However, when the habitat function of both natural soils was tested against that of L soil, dissimilar responses were shown by earthworms. While the R soil was significantly preferred by them, the C soil was significantly avoided (*c.f.*, fig. IV.1), evidencing the limited habitat function of the latter. Considering that the pH measured in the three soils is within the preferred range for *E. andrei* and that this species optimally choose soils with very high OM content (Jänsch et al. 2005) as is seemingly the case of C and R soils, the dissimilar response of earthworms could be related to different intrinsic pedological properties of soils. Some authors (*e.g.,* Natal-da-Luz et al. 2004) had already pointed out that the quality of organic and inorganic fractions of soil may constrain the avoidance behaviour of earthworms. Along with the OM levels, the extremely high silt/clay content of natural soil samples may also compromise the response of earthworms (Jänsch et al. 2005), albeit only in C soil could it act as a combined effect contributing for the decrease of its habitat function. As a result, the C soil was not used for further testing with chemicals to prevent masking effects of pesticides on the avoidance behaviour of earthworms with those constrained by soil properties.

As such, this study strengthens that L soil is obviously not representative of all conditions entailed by different natural soil characteristics, once earthworms preferred the natural soil R. Consequently, the use of a single standard natural soil *per se*, like L, though allowing data reproducibility and comparison between laboratories, it will somehow provide a rough and inaccurate assessment of soil contamination effects as far as it may not estimate overall field scenarios (Amorim et al. 2005, Jänsch et al. 2005). As aforementioned, in order to increase the ecological relevance of the performed avoidance assays with pesticides, the R soil was also used as a substrate to test the toxicity of the formulated compound Viper.

In general, earthworms showed an avoidance response to soils contaminated with a.i. and formulated herbicides, which trend assumed a positive concentration-effect relationship. Besides, it was noticeable that the a.i.s were generally less repellent than the respective formulated compound (*c.f.*, figs. IV.2, IV.3). Notwithstanding, the tested concentrations were far above the recommended application rates of pesticide, meaning therefore, that they would not have a negative impact under realistic situations, while considering the avoidance response of this particular species. Actually, this was already expected considering that herbicides, though biologically active, they are not designed to affect animal species. Consequently, their impairments are likely to occur at higher concentrations than those corresponding to the prescribed spraying rate.

Mikado had significantly constrained the habitat function of L soil for earthworms' at the two highest tested concentrations (*c.f.*, fig. IV.2b). As a matter of fact, these concentrations are beyond the range suggested for the testing of chemicals (ISO 2005), thus the risk represented by this formulated herbicide on *E. andrei* avoidance behaviour is quite low. Similarly, sulcotrione did not represent a risk for earthworm maintenance under concentrations up to the test limit of 1000 mg a.i. $kg^{-1}$ (*c.f.*, fig. IV.2a, table IV.3). Available data indicate a $LC_{50}$ of 1000 mg a.i. $kg^{-1}$ (FOOTPRINT PPDB 2008) for acute exposures of earthworms to sulcotrione. Nevertheless, the soil used as substrate in the tests was not specified. As so, in this situation, the avoidance test was apparently as sensitive as the acute assay with earthworms.

Penoxsulam and its respective formulated product, Viper, induced stronger avoidance behaviour on *E. andrei* than sulcotrione and Mikado, since their avoidance-$EC_{50}$s were remarkably lower (*c.f.,* table IV.3). *E. andrei* was able to detect the presence of penoxsulam and avoid the contaminated L soil at an $EC_{50}$ (*c.f.,* table IV.3) that was at least one order of magnitude lower than the acute-$LC_{50}$ value (> 1000 mg a.i. $kg^{-1}$) determined for *E. fetida* (Dow AgroSciences – Penoxsulam Technical Herbicide Safety Data Sheet), when exposed to the same chemical (the substrate was not specified). On these grounds, the avoidance response seemed to be more sensitive than the acute toxicity endpoint for penoxsulam, although such interpretations should be cautiously taken, since different species and soils were used. Anyway, the apparently higher sensitivity of avoidance tests

relatively to the acute ones has been extensively supported by other authors, along with the reduced ecological relevance of acute earthworm test, and its limited ability to predict or give an early warning of contaminant effects with low costs and effort evolved (Vermeulen et al. 2001, Hund-Rinke et al. 2005, Garcia et al. 2008).

Comparing the effects induced by a.i. vs. formulated product, Mikado and sulcotrione showed similar effects on earthworm behaviour. However, Viper constrained the habitat function of the test soil at lower LOEC (52.7 and 79.0 mg a.i. $kg^{-1}$, for L and R soils, correspondingly) than penoxsulam (LOEC = 100 mg a.i. $kg^{-1}$). Thereby, the behaviour of earthworms was slightly less affected when subjected to the a.i. penoxsulam ($EC_{50}$ = 80.6 mg a.i. $kg^{-1}$) than to the formulated herbicide—Viper—applied on rice fields ($EC_{50}$: 51.5 and 56.9 mg a.i. $kg^{-1}$ on L and R soil, respectively). Indeed, there are published studies indicating that adjuvants, which are added to pesticide formulations as a way to enhance their efficacy, may be responsible for the increased toxicity of the a.i. to certain non-target species (*e.g.,* Tsui and Chu 2003, Cox and Surgan 2006). Although adjuvants are usually omitted from product labels or simply identified as 'inert ingredients', they are biologically or chemically active, and hence able to affect ecological receptors *per se* (Cox and Surgan 2006). Thus, focusing the ecotoxicological profile of new or existing agrochemicals on their a.i. may underestimate the actual toxicity of the formulated product. Our results reinforced the need for a careful assessment of the impacts of formulated products, as it is already established by the regulatory European documents (*e.g.,* EEC 1991). Moreover, this is especially required for the terrestrial compartment so as to fulfil the huge lack of information available for this ecosystem.

Comparing the behaviour of *E. andrei* under different soils contaminated with Viper, a smooth sensitivity difference was observed. Smaller LOEC and $EC_{50}$ values were depicted under L soil than under R soil (*c.f.*, figs. IV.3a, IV.3b, table IV.3). This outcome could be related with the higher OM and clay/silt content determined in R soil, what may constrain pesticide bioavailability (Ying and Williams 2000). Accordingly, Jabusch and Tjeerdema (2005) observed that the soil sorption of penoxsulam occurs both to OM and clay mineral sorption sites. In fact, it is likely that the pedological properties of natural soils may reduce the bioavailability of pesticides (*e.g.*, EC 2003, Römbke et al. 2005, Farenhorst 2006, Garcia et al. 2008), though the properties of some reference standard soils could also be responsible to even lower pesticide bioavailability levels (*e.g.*, artificial soils with higher OM content like the OECD soil). As such, it involves strengthening the use of standard and natural soils in avoidance tests for the testing of chemicals, as a way to achieve robust and feasible responses more closely related with field conditions and its potential overwhelmed impacts (O'Halloran 2006).

Overall, while focusing on indirect effects of stress factors (*e.g.*, chemicals), the avoidance behaviour of earthworms is very promising as a short-term sublethal predictor of detrimental effects

on ecosystem functioning and structure associated to the disappearance of earthworms, which play a major role as soil engineers (Reinecke et al. 2002, Smith et al. 2006). Notwithstanding, the detected concentrations by earthworms were beyond the application rates, which suggests that the risk of these pesticides to edaphic fauna will be low, if application rates are respected. Hence, the avoidance test was seemingly a useful preliminary assessment tool for the tested pesticides. Although some authors refer that the avoidance tests are as sensitive as the reproduction one, the latter should still be performed for the studied herbicides, since a different outcome may be retrieved. Indeed, the reproduction response is based on physiological effects that are not addressed by a behavioural endpoint.

## 4.5 Conclusions

Although LUFA 2.2 is a standard reference soil that has been used for sake of reproducibility and interlaboratorial comparison of tests, this study reinforced that other natural soils should be added for the assessment of chemicals, as the former would never cover all properties entailed by different soils. Regarding the tested herbicides, sulcotrione and Mikado affected the behaviour of earthworms in much less extent than penoxsulam and Viper. On the other hand, the soil contaminated with penoxsulam was avoided in less extent than that contaminated with the formulated herbicide Viper. Such occurrence was possibly related to the increased toxicity represented by the adjuvants added to the commercial products. Additionally, *E. andrei* behaviour was more affected under L soil contaminated with Viper than under R soil, what could rely on the potentially lower bioavailability of the pesticide on the latter substrate probably due to its high OM and clay contents. The tested concentrations, however, were beyond the application rates, which suggests that the risk of these pesticides to edaphic fauna will be low if application rates are respected. Overall, avoidance tests provided a valuable response either to compare the habitat function of different standard and agricultural natural soils or to test the effect of herbicides.

## 4.6 Recommendations and perspectives

An effort should be taken to enlarge the terrestrial ecotoxicological database, namely through the use of different natural soils, as a way to fulfil the huge lack of information available for this ecosystem. Yet, it will also improve the ecological relevance of pesticide assessment on soil environmental compartment, as far as the bioavailability of chemicals would be additionally integrated. In this context, additional research congregating a potential linkage between

physiological activities sustaining the regular metabolism of earthworms and their avoidance behaviour or even their reproductive effects would be welcomed, especially in what regards formulated pesticides. Such approach would provide a robust and comprehensive understanding of chemical effects and would enhance the knowledge behind the avoidance response.

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# Chapter V

## Toxicity evaluation of natural samples from the vicinity of a rice field using two trophic levels

# Toxicity evaluation of natural samples from the vicinity of a rice field using two trophic levels

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## Abstract

An ecotoxicological screening of environmental samples collected in the vicinity of rice fields, followed a combination of physico-chemical measurements and chronic bioassays with two freshwater trophic levels (microalgae: *Pseudokirchneriella subcapitata* and *Chlorella vulgaris*; daphnids: *Daphnia longispina* and *Daphnia magna*). As so, water and sediment/soil elutriate samples were obtained from three sites: (L1) in a canal reach crossing a protected wetland upstream, (L2) in a canal reach surrounded by rice fields, and (L3) in a rice paddy. The sampling was performed before and during the rice culture. During the rice cropping, the whole system quality decreased comparatively to the situation before that period (*e.g.*, nutrient overload, the presence of pesticides in elutriates from sites L2 and L3). This was reinforced by a significant inhibition of both microalgae growth, especially under elutriates. Contrary, the life-history traits of daphnids were significantly stimulated with increasing concentrations of water and elutriates, for both sampling periods.

**Key-words:** rice culture, surface water, elutriates, green algae, daphnids, WET testing, sub-lethal endpoints

## 5.1 Introduction

The worldwide use of agrochemicals represents a crucial contamination source of freshwater ecosystems. In particular, the rice culture further contributes to such contamination scenarios due to its flooded conditions (Miao et al. 2003, Pastor et al. 2004, Padovani et al. 2006), which favours the entrance of contaminants into adjacent watercourses. Hence, agrochemicals can reach the aquatic environment during or after their application, through the drainage of paddy water to the nearby irrigation/drainage ditches, but also via direct overspray, accidental spills, aerial spray drift and/or run-off (Cerejeira et al. 2003, Karpouzas et al. 2005, Padovani et al. 2006, Sánchez et al. 2006).

Rice paddy agro-ecosystems are often located nearby natural protected areas and have been recognised as a contribution to biodiversity maintenance, presenting high ecological resources as surrogate habitats for wetland species (MED-Rice 2003, Miao et al. 2003, Padovani et al. 2006). However, the exposure to non-point source loads of agrochemical residues during the rice crop may pose a risk to non-target aquatic species. Therefore, it is worthwhile to perform regular surveillance programs for the aquatic system, in the vicinity of rice fields. Among the requirements established in the European water policy referred as Water Framework Directive (WFD) (EC 2000) is the monitoring of surface water quality status from each river basin, in order to attain "good" chemical and ecological status as protective goals of the receiving environment. Under this scope, it was developed a toolbox of existing and emerging screening methods for water quality monitoring in support of the implementation of WFD (Roig et al. 2003). In addition to physico-chemical measurements one of the biological assessment tools recommended is the use of whole effluent toxicity (WET) bioassays towards the testing of aqueous samples or sediment extracts (Roig et al. 2003).

Though WET tests present some limitations they are notably useful tools (Chapman 2000), since they integrate interactions occurring in complex mixtures of chemicals, thereby allowing the prediction of potential hazards in the receiving environment (Chapman 2000, Wharfe 2004, Mendonça et al. 2007). This approach has been applied to evaluate different contamination sources affecting water or sediment compartments, through the use of organisms from different trophic levels, such as bacteria, microalgae, cladocerans, macroinvertebrates, sea urchins, bivalves, and fish (*e.g.*, Cheung et al. 1997, Pardos et al. 2000, Anderson et al. 2003, Mucha et al. 2003, Kennedy et al. 2004, Koukal et al. 2004, Losso et al. 2007). For instance, Sánchez et al. (2006) emphasise that similar approaches are especially suited to discern possible effects derived from paddy water discharges.

Beyond the study of the water compartment, the study of the sediment matrix is also indispensable since it is the sink of toxicants. In turn, they can be ressuspended to the water column, through stormwater runoff and water turbulence (Cheung et al. 1997, Viganò et al. 2003), which is likely to occur during the paddy water drainage. In addition, the scrubbing of paddy sediment/soil

particles with adsorbed residues into the drainage ditches (Karpouzas et al. 2005, Padovani et al. 2006) is especially enhanced when the sediment/soil is characterised by a great percentage of silt/clay and organic matter content (Kukkonen and Landrum 1996, Lapota et al. 2000, Viganò et al. 2003), hence justifying the need to evaluate the retention function of the paddy sediment/soil. Considering that the water flux in the drainage ditches is residual, it is likely that the suspended particles with associated pesticides settle in the sediment compartment, thereby becoming a source of contaminants (Viganò et al. 2003). As such, the paddy sediment/soil as well as the sediment from adjacent waterways impose a threat to the aquatic organisms, being more frequently elicited by short-term sub-lethal impairments (Viganò et al. 2003), in spite of most related studies being generally focused on acute responses of non-target individuals.

In this context, the aim of the present work was to evaluate the toxicity of natural samples collected on a paddy field (in-crop assessment) and in an adjacent aquatic system (off-crop assessment) that was a main canal crossing both a protected area upstream and an extensive agricultural area downstream, which is mainly used for rice cropping. Two assessment periods were selected in order to compare the aquatic system quality before and during the rice culture season. According to Johnson et al. (2004), the assessment of impacts on the receiving water requires a battery of test methods and the inclusion of organisms from different trophic levels evidencing variable sensitivity ranges, as a way to achieve reliable and comprehensive information. Therefore, jointly with the physico-chemical scrutiny, chronic WET assays were performed with organisms belonging to two important levels – producers (green microalgae: *Pseudokirchneriella subcapitata* and *Chlorella vulgaris*) and consumers (cladocerans: *Daphnia longispina* and *Daphnia magna*) – responsible for the energy transfer along the freshwater trophic chains (Nyholm and Källqvist 1989, Lampert 1987). In order to enhance the ecological relevance of the study, it was compared the growth and reproductive responses of the autochthonous daphnid, *Daphnia longispina*, and the standard one, *Daphnia magna*. Overall, the combination of physico-chemical measurements with bioassays was performed to accomplish a holistic overview of potential effects on the aquatic ecosystem, triggered by agrochemical exploitation over the rice fields.

## 5.2 Material and Methods

### 5.2.1 Study site, rice culture and sampling design

The Lower Mondego river Valley is located in the centre of Portugal, near Coimbra (40º2'N, 8º43'W). It is one of the most important Portuguese regions of rice production, and it comprises 15000 ha of agricultural land, which is mainly exploited for rice cropping, though corn is also produced but in lower extent. In the proximity of this area there is a wetland – Paul do Taipal - that

was indeed used for rice culture until the 70 decade; nevertheless, in 1999 it was classified, by national regulation (Law by Decree no. 384-B/99, 23.09.1999), as a special protection area for birds, and hence integrated in the Natura 2000 (EEC 1979, EEC 1992, ICN 2008) (code no. PTZPE0040). Furthermore, in 2001, it was integrated on the Ramsar List of Wetlands of International Importance (Ramsar site no. 1107).

The overall hydro-agricultural scheme of the Lower Mondego river Valley is constituted by a widespread irrigation and drainage network (Lima and Lima 2002), being the water flux in the canals/ditches controlled by dams constructed in downstream strategic points. The irrigation water, most of the times, is pumped from the Mondego river, which is the ultimate fate of the outflow from the fields through the drainage canals. Nevertheless, the outflow water is also occasionally used for irrigation.

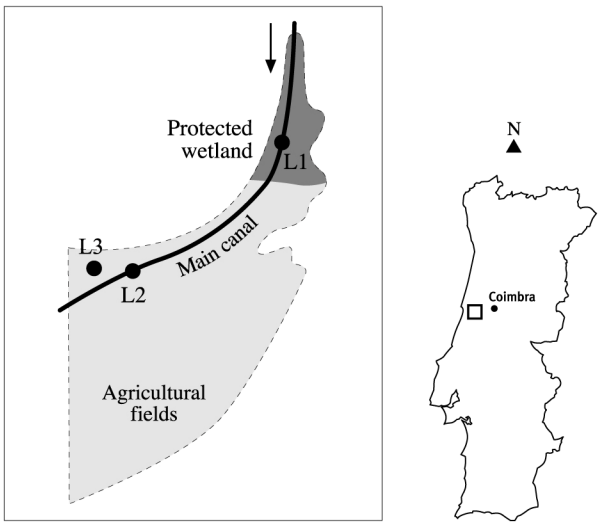


Figure V.1 - Schematic representation of the location of sampling sites (L1, L2 and L3). The arrow indicates the water flux direction. The protected wetland is in dark grey and the nearby agricultural fields are represented in light grey.

The culture of rice starts with land tillage in late April and proceeds until September, when rice is harvested. It grows under discontinuous flow irrigation, being the water depth of 10-15cm. The application of agrochemicals occurs mainly during the end of April up to June, and it is conducted either through terrestrial or aerial spraying. Every time an application is carried out, the paddy water is drained to adjacent canals before pesticide dispersal, being the fields irrigated 1-2 days afterwards. The commonly applied fertiliser is ammonium sulphate. Among the applied pesticides, herbicides are the ones mostly used, such as Stam Novel Flo 480® (480 g propanil $L^{-1}$; EC), Basagran® (480 g bentazone $L^{-1}$; SL), Aura® (200 g profoxydim $L^{-1}$; EC) applied together with Dash HC® (350 g methyl

oleate + methyl palmitate $L^{-1}$; EC), Quitt® (400 g bentazone $L^{-1}$ + 60g $L^{-1}$ MCPA; SL), Facet® (250 g quinclorac $L^{-1}$; SC) and Roundup® (360 g glyphosate $L^{-1}$; SL). Whenever the pests can not be controlled through the dryness of paddy fields farmers make confined applications of the insecticides Quirlan® (24% chlorphenvinfos; SL) or Decis® (25 g deltamethrin $L^{-1}$; EC). However, as far as authors are aware, no insecticides were applied during the rice culture in the year where the sampling was conducted (personal communication of farmers).

Part of the monitoring study was conducted in an irrigation/drainage canal that comes from the protected zone located upstream and crosses the agricultural fields downstream (fig. V.1). Additionally, a site in a rice paddy field was also considered for further surveillance. Although this site presented different hydrodynamic and ecological profiles from those depicted in the drainage/irrigation canal, it was found important to be integrated in this monitoring study to assess the retention function of the paddy sediment/soil and also because its characteristics [*i.e.* very impermeable with high silt/clay (96.5%, table V.1) and organic matter content (4.2 - 4.5%)] represent vulnerable conditions for aquatic environment exposure (MED-RICE 2003), thereby constituting an additional source of contamination that should be assessed.

Therefore, three sampling sites were chosen: in a canal reach crossing the protected wetland (L1), in a canal reach surrounded by rice fields (L2), in a rice paddy (L3) (fig. V.1). Water and sediment/soil samples were collected in two periods (between 2005 and 2006) – before and during rice culture – in each site (except for the site 3, before the rice culture season, since the paddy fields were not flooded for collection of water samples). The chosen sampling periods enabled, on one hand, the assessment of potential impacts due to agrochemical application during the rice cropping season. On the other hand, it allowed the monitoring of the aquatic and sediment conditions before a new rice culture season, and to discern if there was a possible recovery of the overall aquatic system quality, during the rest of the year. The following described procedures were repeated for each sampling period.

### 5.2.2 Collection and preparation of water and elutriate samples

Subsurface water samples were collected to 5L glass containers and transported to the laboratory, where they were stored in the dark at 4ºC, for posterior testing. The sediment (in the canal and paddy field) was sampled with a stainless steal corer, homogenised and sieved in a 2-mm mesh size sieve, according to the U.S.EPA (2001) guidelines. The sediment samples were then maintained in plastic containers covered with aluminium foil and stored under the same conditions as water samples. Considering that the paddy field was dried before the culture season, paddy soil samples were then collected. Composite samples of soils from 3 points were collected in the first 20 cm, after removing the superficial layer of plant debris and humus. The samples were homogenised,

being discarded coarse materials before their sieving through a 2-mm mesh size sieve. The samples thereby obtained were stored in the same way as the sediment samples.

Before testing, water samples from the three sites (L1-W, L2-W and L3-W) were filtered through a 55-µm plankton net. An additional filtration through a GF/C filter was undertaken for water samples to be tested with green algae (U.S.EPA 2002a).

Sediment/soil elutriates were prepared two days before the beginning of bioassays. The followed procedures were adapted from Nebeker et al. (1984), Ankley et al. (1991) and U.S.EPA and U.S.ACE (1998). The sediments/soil samples were mixed with Woods Hole nutritive MBL (Stein 1973) or ASTM hard water (ASTM 1980) culture media for algae and daphnid bioassays, respectively, to a ratio of 1:4 sediment-to-dilution medium. The mixture was placed in an orbital shaker for 2h at ≈ 200 rpm. Afterwards, the samples were allowed to settle overnight. The supernatant (elutriate) was siphoned off and centrifuged at 5000 rpm for 15 min., at 4ºC. The obtained elutriates (L1-Ea, L2-Ea, L3-Ea with ASTM; L1-Eb, L2-Eb, L3-Eb with MBL) were filtered in the same conditions as the water samples for the bioassays with green algae and stored in dark at 4ºC until one-week old.

### 5.2.3 Physico-chemical and microbiological analyses of samples

Concomitantly to the field sampling moments some parameters were measured, such as temperature, pH (pH 330 from WTW), dissolved oxygen ([$O_2$]; Oxi 330 from WTW), conductivity (LF 330 from WTW), through the use of portable water testing meters. The water column transparency was retrieved by the Secchi disc.

In the laboratory, the concentration of Chl *a* ([Chl *a*]) and the total suspended solids (TSS) were determined for water samples (A.P.H.A. 1995). Nutrient analyses were performed by following the Hach test methods for the determination of un-ionised ammonia [$NH_3$-N; the most toxic form for the aquatic organisms (U.S.EPA 1999a, Koukal et al. 2004)], nitrate ($NO_3^-$ -N), nitrite ($NO_2^-$ -N), phosphate ($PO_4^{3-}$) and sulphate ($SO_4^{2-}$) in water and elutriate samples. Furthermore, these samples underwent a chemical analysis after their acidification. The extraction procedure was carried out according to the method no. 3535 for solid-phase extraction published in the SW-846 manual (U.S.EPA 1996). The gas chromatography-mass spectrometry (GC-MS) analysis was performed according to the method no. 8270C, included in the same manual (U.S.EPA 1996). The concentrations of glyphosate were however determined through high-performance liquid chromatography (U.S.EPA 1999b).

Additionally, the density of bacteria in water and elutriate samples collected in the two studied periods was determined as the colony-forming units (CFUs) of bacteria grown in tryptic soy agar (TSA).

The particle-size distribution of sediment samples and its organic matter content (OM) were also analysed according to Buchanan and Kain (1971), being presented the percentage of coarse sand (0.5 – 2 mm) and silt/clay (<63 μm) fractions.

### 5.2.4 Test organisms and rearing conditions

Unialgal inoculum cultures of *Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Chlorella vulgaris* Beijerinck were maintained in 250 mL Erlenmeyer flasks with 100 mL of sterilised MBL in an incubator chamber, with controlled temperature (20±2ºC) and photoperiod ($16^{L}$:$8^{D}$), with light provided by cool-white fluorescent lamps.

*Daphnia longispina* [clone EM7, *sensu* Antunes et al. (2003), isolated from a population collected in Lake Vela, and maintained for several generations in the laboratory] and *Daphnia magna* [clone A, *sensu* Baird et al. (1989a)] were reared in ASTM supplied with an organic additive made of *Ascophylum nudosum* (L.) Le Joli seaweed extract (Baird et al. 1989b), under 20±2 °C and a $16^{L}$:$8^{D}$ photoperiod. Daphnids were fed every two days with *P. subcapitata* at a rate of 1.50 and 3.00 x $10^{5}$ cells $mL^{-1}$ *Daphnia*$^{-1}$ for *D. longispina* and *D. magna*, respectively.

### 5.2.5 WET tests

The tested concentrations 12.5, 25.0, 50.0, 75.0 and 100.0% of water or elutriate samples were prepared by adding MBL or ASTM medium as dilution water, for microalgae and daphnid bioassays, respectively.

*a. Green algae*

Green algae 96h-bioassays were carried out by following the procedures outlined in the U.S.EPA (2002a) and OECD (2002) guidelines. Initial cell densities were approximately $10^{5}$ cells $mL^{-1}$. Besides the treatments already mentioned, an additional one was considered only for the water samples, which consisted in the addition of nutrients to the 100% of water (*i.e.*, 100%+N) in the same concentrations as recommended for MBL medium (U.S.EPA 2002a). It will allow discarding possible growth inhibition due to nutrient limitation. The same treatment was not conduct for elutriates, once they were already made with MBL as dilution medium. Three replicates per treatment were maintained under constant agitation (≈ 100 rpm in an orbital shaker) in the same conditions of algal cultures, with a light intensity ranging between 90.98 and 108.16 μmol $s^{-1}$ $m^{-2}$ (or 4665.64 and 5546.66 lux). *P. subcapitata* and *C. vulgaris* were exposed to water and elutriates from each sampling site and respective period, during 96h. Cell density (counting of cells on a microscope Olympus CKX41 using a Neubauer chamber) was the biomass parameter used for the calculation of the endpoints growth rate (GR, $day^{-1}$) and percentage of growth inhibition (% I).

*b. Daphnids*

Acute bioassays were carried out under U.S.EPA (2002b) procedures. However, since all the tested samples from both sampling periods showed no acute toxicity for both *Daphnia* species, the evaluation proceeded to assess possible sub-lethal effects. Thus, chronic bioassays with daphnids were developed according to OECD (1998), U.S.EPA & U.S.ACE (1998) and U.S.EPA (2002a) guidelines. Neonates less than 24 h old from the third to fifth brood were used to begin the tests. Ten individual replicates were maintained in 50 mL-glass flasks per treatment, being renewed every two days. The general rearing conditions of temperature, photoperiod, feeding rate and additive organic supply, abovementioned, were assured during the chronic tests. The tests were daily monitored for female mortality or offspring production and, whenever present, the neonates were counted and disposed of. Both *D. longispina* and *D. magna* were exposed to water and elutriate samples until 60% or more of surviving control females had three broods. The analysed endpoints either to *D. longispina* or *D. magna* were the somatic growth rate (SGR) and the rate of population increase (*r*). The SGR was calculated from equation (1) (Burns 2000):

$$SGR = [\ln(l_f) - \ln(l_i)] / \Delta t \quad (1)$$

where Δt is the testing interval period in days, $l_f$ and $l_i$ are, respectively, the final and initial body lengths estimated from the moult exopodite measure, according to the allometric relations publish by Pereira et al. (2004). The value of *r* was derived from the Euler-Lotka equation (2) (Meyer et al. 1986):

$$\sum e^{-r.x} . l_x . m_x = 1 \quad (2)$$

where *x* is the age class (days; 0…*n*), $l_x$ is the probability of surviving at age *x,* and $m_x$ is fecundity at age *x.* The standard deviation was determined according to Jackknife technique (Meyer et al. 1986).

### 5.2.6 Data analysis

The point estimate $IC_{10}$ with the 95% confidence limits was calculated by using a Probit analysis (Finney 1971), for the inhibition growth rate percentages of *P. subcapitata* and *C. vulgaris*. A lower effect level was chosen, once it provides a protective estimation of potential deleterious effects on microalgae populations, without compromising the ecological integrity. One-way ANOVAs were performed to find out potential significant differences among values recorded for each tested endpoint (algae GR, daphnids SGR and *r* parameters) under different concentrations of elutriate and water samples. Whenever such differences were found, a Dunnett's test for multiple comparisons of each individual concentration with the control (Zar 1996), was carried out.

## 5.3 Results

As far as physico-chemical parameters of water samples (table V.1) are considered, the pH assumed similar values ranging from 6.8 to 7.9, between sites and sampling periods; while the dissolved oxygen concentration reached a very high level in L3-W (11.5 mg $L^{-1}$) during the rice culture, because of the reduced water depth (10 cm) that allowed constant gaseous exchanges with the air compartment. The highest values of conductivity were recorded during the rice culture especially in L3-W. The transparency was generally reduced (0.2 – 0.4 m) all through the sites and sampling moments. However, the occurrence of higher TSS levels occurred during the rice cropping, in L2-W (0.08 mg $L^{-1}$) and L3-W (0.20 mg $L^{-1}$) samples. The highest concentration of Chl *a* (59.0 µg $L^{-1}$) was determined in L2-W, for the same culture season.

Particle-size distribution analysis of sediments showed the dominance of coarse sand in sites L1 (68.0%) and L2 (62.7%), whilst in site L3 the predominant fraction was of silt/clay particles (96.5%), which is in straight agreement with its enhanced organic matter content (4.18 - 4.52%) relatively to the sites L1 and L2 (2.02 - 2.16 and 2.81 - 3.12%, respectively), during the rice crop. CFUs values, particularly for L1-W, both before (4.6 x $10^3$ CFU $mL^{-1}$) and during the rice cropping (6.0 x $10^4$ CFU $mL^{-1}$) (table V.1). Notwithstanding, higher bacterial densities occurred during the latter season for water or elutriate samples, independently of the site. Within elutriates, those made with MBL favoured the development of bacterial colonies.

As it can be observed in table V.1, it was quite evident the extremely high concentrations of un-ionised ammonia in elutriate samples, mainly during the rice crop and especially for those made with MBL medium [L1-Ea: 1.05 – 1.72 mg $L^{-1}$ $NH_3$-N ***vs.*** L1-Eb: 2.43 – 9.72 mg $L^{-1}$ $NH_3$–N; L2-Ea: 3.41 – 6.68 mg $L^{-1}$ $NH_3$–N ***vs.*** L2-Eb: 4.82 – 5.60 mg $L^{-1}$ $NH_3$–N]. Likewise, the levels of un-ionised ammonia and nitrite in water samples were more elevated during the rice culture (0.49 - 0.85 mg $L^{-1}$ $NH_3$–N and 0.20 – 0.40 mg $L^{-1}$ $NO_2^-$-N, respectively) than before it (0.28 - 0.35 mg $L^{-1}$ $NH_3$–N and 0.07 - 0.09 mg $L^{-1}$ $NO_2^-$-N, respectively), being their highest values recorded for site L2 (L2-W), independently of the sampling period. On the other hand, the nitrate concentrations assumed their outmost peaks before the rice culture in elutriate samples made with MBL (6.80 – 7.00 mg $L^{-1}$ $NO_3^-$-N), although they were also high during the rice cropping (2.90 - 4.70 mg $L^{-1}$ $NO_3^-$-N). In what concerns the water samples, they presented lower nitrate concentrations, which were similar between sites and did not much differ among sampling periods.

The phosphate concentrations were considerably high before the rice culture in elutriates L1-Ea, L2-Ea, L1-Eb and L2-Eb (0.12, 0.17, 0.08 and 0.10 mg $L^{-1}$ $PO_4^{3-}$, respectively), while during the rice season it was attained their peak in L2-Ea (0.20 mg $L^{-1}$ $PO_4^{3-}$). In water samples, the phosphates were generally higher during the rice cropping sampling period, especially in L1-W and L2-W samples (0.28

Table V.1 - Physico-chemical parameters determined for both sampling periods in different types of sample from sites L1, L2 and L3.

| Parameters | Sample type | Before rice culture | | | During rice culture | | |
|---|---|---|---|---|---|---|---|
| | | L1 | L2 | L3 | L1 | L2 | L3 |
| pH | Water | 7.1 | 7.8 | NA | 6.8 | 7.2 | 7.9 |
| $[O_2]$ (mg $L^{-1}$) | Water | 6.2 | 7.8 | NA | 6.7 | 6.0 | 11.5 |
| Temperature (ºC) | Water | 15.0 | 15.0 | - | 22.0 | 22.0 | 28.2 |
| Conductivity (µS $cm^{-1}$) | Water | 653 | 589 | NA | 1072 | 861 | 1359 |
| Transparency (m) | Water | 0.2 | 0.4 | NA | 0.3 | 0.2 | 0.1* |
| [Chl a] (µg $L^{-1}$) | Water | 13.7 | 6.3 | NA | 22.0 | 59.0 | 25.0 |
| TSS (mg $L^{-1}$) | Water | 0.06 | 0.06 | NA | 0.05 | 0.08 | 0.20 |
| % coarse sand (0.5-2 mm) | Sediment | NA | NA | NA | 68.0 | 62.7 | 1.0 |
| % silt/clay (<63 µm) | Sediment | NA | NA | NA | 7.3 | 26.3 | 96.5 |
| % OM | Sediment/soil | 2.02 | 2.81 | 4.18 | 2.16 | 3.12 | 4.52 |
| CFU.$mL^{-1}$ | Water | 4.6E+03 | 2.8E+03 | NA | 6.0E+04 | 3.7E+04 | 2.2E+04 |
| | Elutriate a | 4.0E+02 | 4.7E+02 | 3.0E+02 | 2.8E+03 | 1.4E+03 | 1.2E+03 |
| | Elutriate b | 7.0E+02 | 4.8E+02 | 4.2E+02 | 2.2E+04 | 4.8E+03 | 1.5E+03 |
| Nutrients (mg $L^{-1}$) | | | | | | | |
| $NH_3$-N | Water | 0.28 | 0.35 | NA | 0.49 | 0.85 | 0.54 |
| | Elutriate a | 1.05 | 3.41 | 0.14 | 1.72 | 6.68 | 1.03 |
| | Elutriate b | 2.43 | 4.82 | 0.13 | 9.72 | 5.60 | 0.93 |
| $NO_3^-$-N | Water | 0.01 | 0.02 | NA | 0.01 | 0.01 | 0.01 |
| | Elutriate a | 0.10 | 0.40 | 0.20 | 0.10 | 0.30 | 0.40 |
| | Elutriate b | 6.80 | 6.80 | 7.00 | 2.90 | 4.00 | 4.70 |
| $NO_2^-$-N | Water | 0.07 | 0.09 | NA | 0.20 | 0.40 | 0.20 |
| | Elutriate a | 0.01 | 0.01 | 0.01 | 0.00 | 0.01 | 0.02 |
| | Elutriate b | 0.20 | 0.28 | 0.01 | 0.38 | 0.31 | 0.13 |
| $PO_4^{3-}$ | Water | 0.08 | 0.14 | NA | 0.28 | 0.27 | 0.21 |
| | Elutriate a | 0.12 | 0.08 | 0.04 | 0.00 | 0.20 | 0.12 |
| | Elutriate b | 0.17 | 0.10 | 0.04 | 0.01 | 0.01 | 0.03 |
| $SO_4^{2-}$ | Water | 39 | 43 | NA | 68 | 64 | 76 |
| | Elutriate a | 236 | 228 | 236 | 132 | 138 | 114 |
| | Elutriate b | 59 | 70 | 46 | 26 | 32 | 38 |

NA – Not available. Elutriate a and Elutriate b were made with ASTM and MBL dilution media, respectively. $[O_2]$ (dissolved oxygen); [Chl a] (concentration of Chl a); TSS (total suspended soils); OM (organic matter content). (*) Corresponds to the water depth in site 3.

and 0.27 mg $L^{-1}$ $PO_4^{3-}$, respectively). For the same sampling period, water samples presented relatively high concentrations of sulphates (64 – 76 mg $L^{-1}$ $SO_4^{2-}$), while elutriates made with ASTM showed even higher values due to the calcium sulphate used to prepare that synthetic medium.

Table V.2 - Concentrations of pesticides (ng $L^{-1}$) quantified in elutriates made with ASTM (Elutriate a) and MBL (Elutriate b), from sediments collected in sites L1, L2 and L3, during the rice culture.

| Pesticides | Sample | Sites | | |
|---|---|---|---|---|
| | | L1 | L2 | L3 |
| *m*-Chloroaniline | Elutriate a | < 0.20 | 5.21 | 6.42 |
| | Elutriate b | < 0.20 | 3.26 | 5.32 |
| Alachlor | Elutriate a | < 0.20 | 4.91 | < 0.20 |
| | Elutriate b | < 0.20 | 4.71 | < 0.20 |
| Glyphosate | Elutriate a | < 0.20 | 5.34 | 6.55 |
| | Elutriate b | < 0.20 | 3.37 | 5.41 |
| Carbofuran | Elutriate a | < 0.20 | 4.96 | 5.64 |
| | Elutriate b | < 0.20 | 5.89 | 6.12 |

The chemical characterisation of samples did not detect pesticides in water samples from both sampling periods and for all sites, as well as in elutriate samples obtained before the culture period. In contrast, during the rice culture season the pesticides were quantified in elutriates from L2 and L3 either made with ASTM or MBL, though L3-Ea and L3-Eb presented slightly higher concentrations of glyphosate (5.41 – 6.55 ng $L^{-1}$), *m*-chloroaniline (5.32 – 6.42 ng $L^{-1}$), and the insecticide carbofuran (5.64 – 6.12 ng $L^{-1}$), than L2-Ea or L2-Eb (3.37 - 5.34, 3.26 - 5.21 and 4.96 - 5.89 ng $L^{-1}$, respectively).

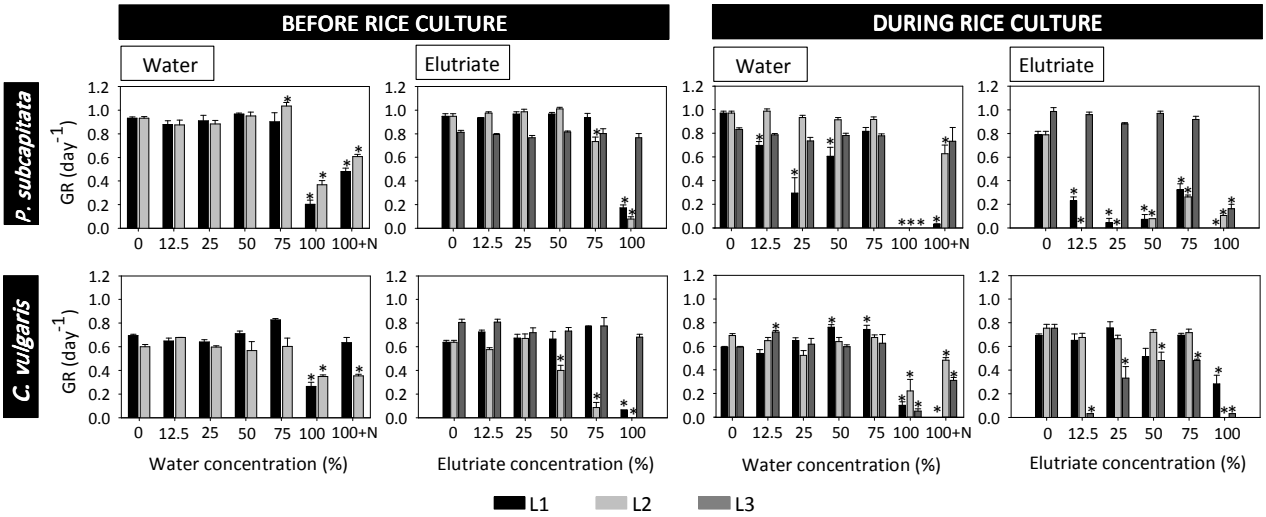


Figure V.2 - Growth rates (GR; $day^{-1}$) of *P. subcapitata* and *C. vulgaris* exposed to different concentrations of water samples (nutrients added in the "100+N" treatment) and elutriate from sites L1, L2 and L3, before and during the rice crop. Error bars represent standard error and * indicates a value significantly different from the control ($P < 0.05$).

Table V.3 - Statistical outcome of one-way analysis of variance for microalgae (*P. subcapitata* and *C. vulgaris*) growth rate (GR) and the life-history endpoints [somatic growth rate (SGR) and intrinsic population increase (*r*)] of daphnids (*D. longispina* and *D. magna*) exposed to water samples (W) and elutriates (E) from sites L1 (L1-W, L1-Ea, L1-Eb), L2 (L2-W, L2-Ea, L2-Eb) and L3 (L3-W, L3-Ea, L3-Eb), before and during the rice cropping season.

| | | | *P. subcapitata* | | | *C. vulgaris* | | | | | *D. longispina* | | | *D. magna* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sampling period | Sample | Endpoint | *F* | *d.f.* | *P* | *F* | *d.f.* | *P* | Sample | Endpoint | *F* | *d.f.* | *P* | *F* | *d.f.* | *P* |
| Before rice season | L1-W | GR | 61.495 | 6,16 | <0.001 | 40.318 | 6,15 | <0.001 | L1-W | SGR | 1.883 | 5,52 | 0.113 | 1.441 | 5,54 | 0.255 |
| | | | | | | | | | | *r* | 98.814 | 5,52 | <0.001 | 6.252 | 5,54 | <0.001 |
| | L2-W | GR | 69.788 | 6,17 | <0.001 | 12.827 | 6,17 | <0.001 | L2-W | SGR | 5.185 | 5,52 | <0.001 | 8.106 | 5,51 | <0.001 |
| | | | | | | | | | | *r* | 5.635 | 5,53 | <0.001 | 17.040 | 5,52 | <0.001 |
| | L1-Eb | GR | 129.957 | 6,17 | <0.001 | 52.469 | 5,14 | <0.001 | L1-Ea | SGR | 7.724 | 5,54 | <0.001 | 2.196 | 5,53 | 0.068 |
| | | | | | | | | | | *r* | 16.819 | 5,54 | <0.001 | 7.748 | 5,53 | <0.001 |
| | L2-Eb | GR | 201.065 | 6,17 | <0.001 | 113.127 | 6,16 | <0001 | L2-Ea | SGR | 33.604 | 5,54 | <0.001 | 6.832 | 5,54 | <0.001 |
| | | | | | | | | | | *r* | 165.610 | 5,54 | <0.001 | 10.436 | 5,54 | <0.001 |
| | L3-Eb | GR | 0.595 | 6,17 | 0.730 | 2.489 | 6,14 | 0.075 | L3-Ea | SGR | 5.077 | 5,54 | <0.001 | 5.240 | 5,54 | <0.001 |
| | | | | | | | | | | *r* | 16.819 | 5,54 | <0.001 | 12.026 | 5,54 | <0.001 |
| During rice season | L1-W | GR | 41.125 | 6,14 | <0.001 | 161.053 | 6,16 | <0.001 | L1-W | SGR | 4.045 | 5,50 | 0.004 | 2.186 | 5,54 | 0.069 |
| | | | | | | | | | | *r* | 3.378 | 5,50 | 0.010 | 4.957 | 5,54 | <0.001 |
| | L2-W | GR | 122.638 | 6,14 | <0.001 | 14.791 | 6,15 | <0.001 | L2-W | SGR | 6.690 | 5,54 | <0.001 | 3.967 | 5,54 | 0.004 |
| | | | | | | | | | | *r* | 15.473 | 5,54 | <0.001 | 16.022 | 5,54 | <0.001 |
| | L3-W | GR | 40.736 | 6,14 | <0.001 | 46.614 | 6,15 | <0.001 | L3-W | SGR | 2.767 | 5,54 | 0.027 | 3.792 | 5,53 | 0.005 |
| | | | | | | | | | | *r* | 15.189 | 5,54 | <0.001 | 10.579 | 5,54 | <0.001 |
| | L1-Eb | GR | 96.522 | 6,13 | <0.001 | 35.740 | 6,16 | <0.001 | L1-Ea | SGR | 9.856 | 5,54 | <0.001 | 5.040 | 5,54 | <0.001 |
| | | | | | | | | | | *r* | 52.877 | 5,54 | <0.001 | 8.555 | 5,54 | <0.001 |
| | L2-Eb | GR | 454.958 | 3,7 | <0.001 | 59.947 | 6,16 | <0.001 | L2-Ea | SGR | 19.937 | 5,54 | <0.001 | 17.803 | 5,54 | <0.001 |
| | | | | | | | | | | *r* | 20.285 | 5,53 | <0.001 | 25.518 | 5,54 | <0.001 |
| | L3-Eb | GR | 134.803 | 6,13 | <0.001 | 10.616 | 4,11 | <0.001 | L3-Ea | SGR | 7.973 | 5,54 | <0.001 | 11.744 | 5,54 | <0.001 |
| | | | | | | | | | | *r* | 59.891 | 5,54 | <0.001 | 66.586 | 5,54 | <0.001 |

Ea – elutriate made with ASTM; Eb – elutriate made with MBL.

The herbicide alachlor (4.91 – 4.71 ng $L^{-1}$) was only detected in elutriate samples gathered from site L2 (L2-Ea and L2-Eb) (table V.2). No pesticides were determined for L1-Ea and L1-Eb elutriates during the rice culture.

In general, the bioassays with microalgae met the performance criteria specified in the pursued guidelines. The final results showed that water, but mainly elutriate samples collected during the rice cropping period, induced more significant inhibition occurrences on *P. subcapitata* and *C. vulgaris* GR, than those obtained before it (fig. V.2, table V.3). This is in accordance with the generally lower $IC_{10}$s estimated for *P. subcapitata* exposed to L1-W (<12.5%), L1-Eb and L2-Eb (<12.5%), and for *C. vulgaris* exposed to L3-Eb (<12.5%) samples, gathered during the rice cropping (table V.4). Before this season, the highest concentrations of water (100%) and elutriates (50-100%) from sites L1 and L2 had significantly depleted the growth of both species, especially that of *C. vulgaris* exposed to concentrations ≥ 50.0% L2-Eb ($IC_{10}$ = 22.3%) (fig. V.2, table V.4).

Notwithstanding, throughout the performed assays the significant reductions of microalgae growth denoted, at least, under the 100% of natural samples were generally maintained when

Table V.4 - $IC_{10}$ (% of water and elutriate) values determined to the percentage of growth rate inhibition, for *P. subcapitata* and *C. vulgaris* exposed to water (W) samples and elutriates (Eb – with MBL) from sites L1 (L1-W, L1-Eb), L2 (L2-W, L2-Eb) and L3 (L3-W, L3-Eb), before and during the rice cropping season.

| | | Microalgae species | |
|---|---|---|---|
| Sampling period | Sample | *P. subcapitata* | *C. vulgaris* |
| Before rice season | L1-W | 49.1 | 31.6 |
| | | - | (21.61 - 39.79) |
| | L2-W | 34.7 | 53.7 |
| | | (-16.77 - 59.42) | - |
| | L1-Eb | 70.5 | 56.5 |
| | | - | (-2260.22 - 82.30) |
| | L2-Eb | 68.8 | 22.3 |
| | | (63.75 - 72.33) | (-11.21 - 37.10) |
| | L3-Eb | NT | 76.6 |
| | | - | - |
| During rice season | L1-W | < 12.5 | 22.3 |
| | | - | - |
| | L2-W | 58.5 | 32.0 |
| | | - | - |
| | L3-W | 41.2 | 40.2 |
| | | - | (29.94 - 48.22) |
| | L1-Eb | < 12.5 | 29.1 |
| | | - | (-48.63 - 54.20) |
| | L2-Eb | < 12.5 | 38.4 |
| | | - | - |
| | L3-Eb | 48.5 | < 12.5 |
| | | - | - |

(-) Not obtained. (NT) No toxicity.

nutrients were added to the treatments 100%+N (fig. V.2). It indicated that nutrients in plain samples were not limiting algae growth.

Considering the calculated $IC_{10}$s (table V.4), *P. subcapitata* was slightly more sensitive during the rice cropping, whilst *C. vulgaris* was less tolerant before the rice cropping season. Overall, samples from sites L1 and L2 induced more noticeable impairments in algae GR than those coming from site L3, except regarding elutriate samples from the latter site, during the agricultural season, which present significant toxic effects for *C. vulgaris*.

In what concerns the WET tests with daphnids, the validity criteria addressed in the followed guidelines were accomplished. The results indicated that there were no inhibitory responses either for *D. longispina* or *D. magna* (figs. V.3, V.4, table V.3). Indeed, *r* was the most sensitive parameter,

since it was significantly stimulated with the increase of water and/or elutriate concentrations for both species (fig. V.4, table V.3). On the other hand, it is clear that the SGR of *D. longispina* and *D. magna* was more significantly stimulated under elutriate samples, especially Ea2 and Ea3, from both sampling periods (fig. V.3, table V.3). Nevertheless, in a general view, regarding water and elutriate samples, the average values recorded for *r* were slightly higher during the rice season for *D. longispina* and *D. magna* (fig. V.4), whereas, the endpoint SGR was somewhat enhanced only for *D. longispina* (fig. V.3).

Overall, the differences between the two sampling moments and daphnid species responses were not remarkably noticeable.

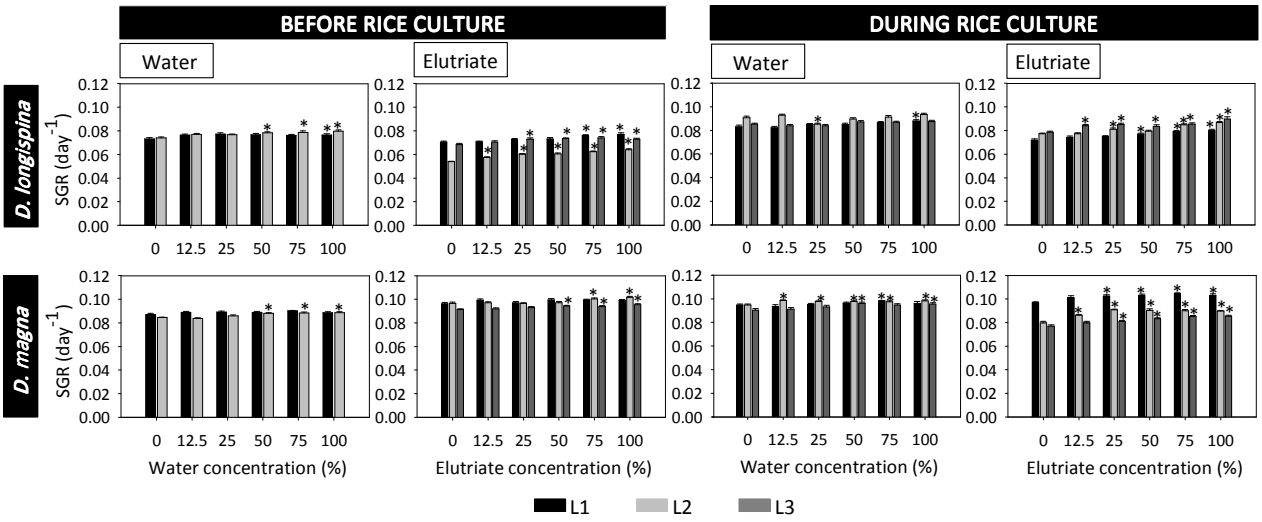


Figure V.3 - Somatic growth rate (SGR; day$^{-1}$) of *D. longispina* and *D. magna* exposed to different concentrations of water and elutriate samples from sites 1, 2 and 3, collected before and during the rice culture. Error bars represent standard error and * indicates a value significantly different from the control ($P < 0.05$).

## 5.4 Discussion

In a general view, the physico-chemical characterisation as well as the algae bioassay responses clearly pointed out for a harmful change in the off-crop and in-crop environment quality during rice culture, comparatively to that of the period before this cropping season. These changes may probably be triggered by the overloading of nutrients and pesticides contamination reaching water and sediment/paddy soil compartments, being the seasonal variation most likely due to overall agricultural practices, namely the application of agrochemicals. Indeed, several authors documented that the environmental occurrence of fertiliser and pesticide residues was restricted to the cropping

season or to intermittent physical events (*e.g.*, strong rainfall, drainage of fields) (Albanis et al. 1998, Santos et al. 2000, Cerejeira et al. 2003, Padovani et al. 2006).

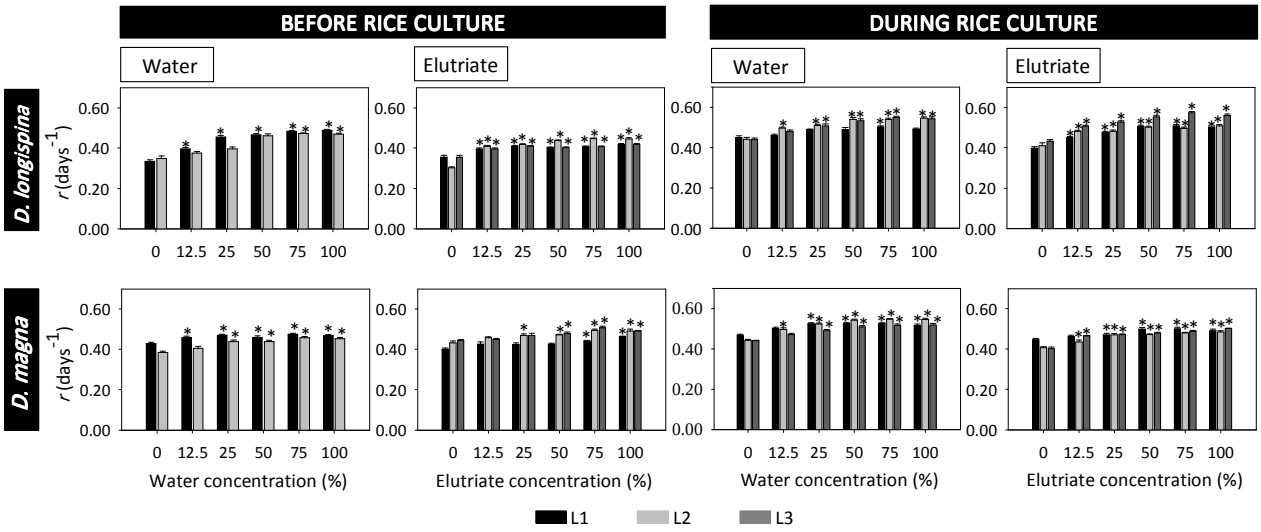


Figure V.4 - Population intrinsic rate ($r$; days$^{-1}$) of *D. longispina* and *D. magna* exposed to different concentrations of water and elutriate samples from sites 1, 2 and 3, collected before and during the rice culture. Error bars represent standard error and * indicates a value significantly different from the control ($P$ < 0.05).

During the rest of the year, however, the whole system was allowed to recover, which was generally evidenced by the lower nutrient content, absence of pesticides and reduced bacterial density for all sites and studied environmental compartments (tables V.1, V.2), measured before the rice cropping.

### 5.4.1 Physico-chemical characterisation

The overuse of fertilisers enhances the occurrence of non-point inputs of huge amounts of nutrients into nearby watercourses (Smith et al. 1999). Similarly, the highest nutrient concentrations in this study, namely regarding un-ionised ammonia and sulphates, were detected in all water samples during the rice cropping season, what could be attributed to the application of ammonium sulphate fertilisers. The determined physico-chemical parameters for both seasons were below the allowable thresholds outlined in national legislation, for surface water quality or irrigation water (MA 1998). Nevertheless, the levels of nitrogen compounds (*i.e.*, un-ionised ammonia, nitrates and nitrites) and phosphates, particularly for L1-W and L2-W during the cropping season (*c.f.*, table V.1) were within the range of total N (TN) and P (TP) defined for mesotrophic (0.70 – 1.50 mg TN L$^{-1}$) and

eutrophic (> 0.075 mg TP $L^{-1}$) states of streams, respectively (Dodds et al. 1998). The nutrient enrichment of streams is typically accompanied by increases in algal yields (Smith et al. 1999). Hence, the elevated concentrations obtained for suspended Chl *a* during the rice culture, most of all in site L2, underpin those meso- and eutrophic (10 - 30 µg $L^{-1}$ and > 30 µg $L^{-1}$ of Chl *a*, respectively) conditions already mentioned (Dodds et al. 1998). These advanced trophic states are further corroborated by the low water column transparency (≤ 0.3 m), the strongly enhanced bacterial density and relatively high conductivity and TSS values, generally verified in all the water samples collected during the rice crop.

In spite of this organic contamination profile, none of the pesticides applied to the rice fields were quantified in water samples, for all sampling sites and periods. This could be assigned to their rapid transformation under field conditions (*e.g.*, sunlight, soil properties, application rates, microbial activity, pesticide chemical and environmental stability), as already mentioned for propanil (Santos et al. 2000, Tomlin 2000), profoxydim (Sánchez et al. 2006), MCPA (González et al. 2006), quinclorac (Tomlin 2000, Resgalla et al. 2007), bentazone and glyphosate (Tomlin 2000).

In what concerns elutriates, the measured nutrient content was quite conspicuous relatively to the water samples, albeit the highest records were, once again, perceived during the rice crop for all sites, particularly for the nitrogenous compounds and sulphates. In part, this is the contribution of nutrients coming from the dilution media MBL and ASTM, respectively, but also from the potential release of organic compounds adsorbed to the sediment particles (Mucha et al. 2003), as a result of fertiliser applications. Concomitantly, the considerable high organic matter content of sediments and the paddy soil, mainly during the rice culture (table V.1), along with an increased bacterial density responsible for its further decaying, could have also enhanced the concentration of nutrients released to elutriates (Lapota et al. 2000).

Contrary to water samples, elutriates evidenced the presence of pesticides, though they were only detected during the rice culture in sites L2 and L3. Site 1 was always free of pesticides, which is in accordance with the protection goals for its surrounding protected area. However, in its vicinity, concentrations of *m*-chloroaniline, glyphosate and carbofuran were quantified, being slightly higher in L3-E than in L2-E, for both dilution media (table V.2). Thus, the paddy soil presented a rough surplus of pesticides what is expected to happen as it is the main target area for their application. Furthermore, a higher content of silt/clay (96.5%) measured in the paddy soil, tends to increase the amount of organic matter bound to its particles, which, in turn, may enhance the adsorption of sediment-bound organic contaminants (Kukkonen and Landrum 1996). Nevertheless, the incidence of alachlor was only recorded in L2-Ea and L2-Eb samples obtained from sediments collected in the main canal. This aspect together with the determination of carbofuran were beyond authors' expectation, but farmers often mix or add extra agrochemicals to attain an efficient control of weeds

or pests, as a way to increase crop production yields. *m*-Chloroaniline is one of the degradation products of propanil (Konstantinou et al. 2001) – an herbicide extensively applied in the study area that is rapidly transformed under field conditions (Tomlin 2000), as aforementioned. Alachlor, glyphosate and carbofuran have considerable half-lives ($DT_{50}$) in soil ranging between 18-20 days, 3-174 days and 20-60 days, respectively (Albanis et al. 1998, Tomlin 2000). Consequently, these $DT_{50}$s indicate, on one hand, that the pesticide residues are completely degraded until the beginning of the next culture season, therefore justifying their absence in the first sampling period and confirming the possible system recovery. On the other hand, they are sufficient long to enhance the likelihood of their entrance in the nearby ditches through the drainage of fields along the rice culture.

### 5.4.2 Bioassays with microalgae

The sub-lethal responses obtained with microalgae were somehow dissimilar between the two sampling periods. It was noticed that either *P. subcapitata* or *C. vulgaris* growth rates were more significantly inhibited during the rice production than before it, which is in straight compliance with the general physico-chemical degradation already described for the different environmental matrices, during that sampling period. Notwithstanding, in a broad sense, elutriates were more toxic for green microalgae than water samples, for both sampling periods, though this was clearer during the rice cropping. Actually, the area surrounding the study site has been used for agricultural production since many years, which could possibly lead to historic contamination mainly associated to the sediment/soil.

As so, during the rice season, it was quite evident that elutriates L1-Eb and L2-Eb were strongly deleterious for *P. subcapitata* under all the tested dilutions, whilst the toxicity of L3-Eb was observed only for its 100%. On the other hand, L3-Eb was the most toxic elutriate sample for *C. vulgaris* growth rate, followed by L1-Eb and L2-Eb (*c.f.*, table V.4). Indeed, L3 was the primary target of agricultural practices involving the direct application of agrochemicals. Among the pesticides detected in L2-Eb and L3-Eb were measured two herbicides that could represent a risk for algae growth – glyphosate (in L2-Eb and L3-Eb), while the determination of alachlor was confined to L2-Eb (table V.2). However, according to the toxicity values referred in literature, the measured amounts were seemingly unable to induce a harmful effect on green algae [**alachlor**: 96-h $EC_{50}$ = 6 µg $L^{-1}$ (Fairchild et al. 1997) and 72-h $EC_{50}$ = 12 µg $L^{-1}$ (Tomlin 2000) for *P. subcapitata*; 24-h $EC_{50}$ = 37.8 µg $L^{-1}$ for *Scenedesmus vacuolatus* (Junghans et al. 2003). **Glyphosate**: 72-h $EC_{50}$ = 485 mg $L^{-1}$ (Tomlin 2000) and 24-h $EC_{10}$ = 92.5 mg $L^{-1}$ (Cedergreen and Streibig 2005) for *P. subcapitata*]. In spite of this, there is a chance that the mixture of chemicals and respective by-products may induce synergistic effects under low environmental concentrations (Cedergreen and Streibig 2005).

Furthermore, the toxicity elicited by elutriates could be also related with their highest content of un-ionised ammonia essentially recorded in L1-Eb and L2-Eb, during the culture season. Furthermore, the considerable bacterial densities determined in L1-Eb ($2.2x10^4$ CFU $mL^{-1}$) and L2-Eb (4.8x103 CFU $mL^{-1}$) envisaged their enhanced activity on decaying organic matter probably released from the sediments, what could possibly be a contribution for the raising of un-ionised ammonia content (Lapota et al. 2000). Although ammonia is a recommended nitrogen source for algae growth (Mayer et al. 1998), under certain circumstances (*e.g.,* pH increase), there is a chance that the ion ammonium ($NH_4^+$) dissociates to form un-ionised ammonia ($NH_3$), which had been demonstrated since long time to exert negative effects on microalgae growth rate (*e.g.*, Azov and Goldman 1982, Källqvist and Svenson 2003). Particularly, the toxicity of ammonia associated to sediment and soil compartments was observed by Azov and Goldman (1982) for the photoassimilation of $^{14}C$ by *Scenedesmus obliquus* under exposures to 8.5 - 34.0 mg $NH_3$ $L^{-1}$. Cheung et al. (1997) mentioned the growth inhibition of the diatom *Skeletonema costatum* exposed to average ammonia levels ranging between 8.4 and 18.0 mg ammonia-N $L^{-1}$ in sediment elutriates. In a similar approach designed for elutriate assessment, Mucha et al. (2003) considered that the estimated concentration of ≈ 68.0 µg $NH_3$-N $L^{-1}$ in 8.3 mg $L^{-1}$ of total ammonia-N contributed for the growth rate depletion of the diatom *Phaeodactylum tricornutum*.

The toxicity of water samples, during the rice cropping, was mostly associated to the 100% concentration of L2-W and L3-W samples for both species, being remarkably higher for *P. subcapitata* subjected to L1-W sample. Considering that no pesticides were detected in L2-W and L3-W, there could be other unmonitored xenobiotics inhibiting algae growth. Accordingly, in a general view, the surplus of nutrients added to the water samples had only attenuated the observed growth decrease, being still identified significant inhibitions relatively to the control.

The site L1, located in the canal reach that crosses the protected wetland, is often subjected to a residual water flux that most of the time it is totally absent. Additionally, there is a dense macrophyte cover in that zone which also extends along the canal margins and it is mainly characterised by *Typha latifolia*, *Scirpus lacustris* and *Phragmites australis*. According to Ervin and Wetzel (2003), low water velocities combined with wetland macrophytes allow the accumulation of dead biomass and the concentration of allelochemicals. As a matter of fact, the allelopathic inhibition of phytoplankton due to the release of toxic chemical substances by macrophytes, like *Typha* sp., *Juncus* sp. and *Phragmites* sp., was pointed out in some related studies (*e.g.*, Ervin and Wetzel 2003). Hence, one explanation for the impairment of *P. subcapitata* exposed to L1-W could be linked to the likely presence of allelochemicals.

Before the rice culture, the overwhelming scenario that has been so far described was generally softened for both species. The inhibitory responses recorded under exposures of water

samples from L1 and L2 were restricted to the 100% treatment and were less steep (fig. V.2). This was coherent with the lowering of nutrients' content, conductivity and bacterial density. Notwithstanding, it was noticed once more that the addition of nutrients did not prevent significant reductions on microalgae growth, except for *C. vulgaris* exposed to L1-W 100%+N. Even though, the nutrients had clearly attenuated a potential negative effect occurring in the 100% water, possibly through the improvement of algae tolerance to stressors (Moreira-Santos et al. 2004).

Regarding the effects of elutriates before the rice culture, the sample L2-Eb denoted higher toxicity, especially for *C. vulgaris* (table V.4), what could be, once again, linked to the conspicuous concentration of un-ionised ammonia (table V.1) measured before the rice culture. Actually, it is quite difficult to discern possible cause-effect relationships, as a multitude of physical, chemical and biological components in a complex mixture can be interacting in a synergistic, antagonistic and/or additive way, to bring about a harmful effect onto several functions of the test organisms (Pardos et al. 1998).

In a general view, the response pattern of both algae was similar, being the major differences in their sensitivity denoted during the agricultural season. Such differential sensitivity of the algae species is actually in line with the outcomes of other studies (*e.g.*, Nyholm and Källqvist 1989, de Figueiredo et al. 2004). Thus, the best strategy when assorting natural samples' toxicity through the use of algae tests is to include more than one species.

### 5.4.3 Bioassays with daphnids

Contrary to algae responses, the daphnids were more tolerant showing a significant stimulatory trend of the somatic growth and population increase endpoints, when exposed either to water samples or elutriates, from all the sites and sampling periods (figs. V.3, V.4, table V.3). Similar studies aimed in evaluating the toxicity of contaminated water and sediment elutriates found that microalgae were more sensitive than daphnids (*e.g.*, Koukal et al. 2004, Ra et al. 2007). Even though, the ecological relevance of chronic bioassays is enhanced when directly related trophic levels, such as algae and daphnids, are exposed to the same contamination source (Podemski and Culp 2001). In this way, both trophic levels may be affected by the same stressors interacting in the receiving environment, which may provide an overview of the potential change in the nutritional quality of available diet for consumers, what as well may constrain their adaptation responses when exposed to xenobiotics (Podemski and Culp 2001).

The occurrence of stimulatory responses on cladoceran life-history traits had already been reported in other bioassays carried out with natural samples (*e.g.*, Antunes et al. 2007a, Antunes et al. 2007b). Several authors sustain that daphnids can improve their fitness through the grazing of dissolved or suspended nutrients in water column (Lampert 1987, Roche 1998, Nandini et al. 2005)

or through feeding on particulate sediment-bounded organic matter often released to elutriates (Viganò et al. 2003, Antunes et al. 2007b). Besides, the generalist feeding behaviour of cladocerans also allow them to feed on available bacteria and some algae (Lampert 1987, Stewart and Konetsky 1998), which are often represented in great amounts on natural samples. In fact, the tested environmental samples evidenced high TSS (for water samples), nutrient and bacterial contents, which was generally overwhelmed during the rice culture, thereby sustaining the enhancement of the average values determined for SGR and *r* of *D. longispina* and *D. magna*. Yet, there was no much difference between the autochthonous and the standard cladoceran responses. Above all, the population increase was the most significantly outperformed, thus sensitive, endpoint for both species, irrespective of the sampling period.

Within the recorded pesticides in sediment elutriates, during the rice season, the insecticide carbofuran was the one that could promote stronger negative effects on cladocerans at low concentrations. However, the obtained levels were below the toxicity thresholds found in literature for *D. magna* [*e.g.*, 48-h $LC_{50}$ = 38.6 µg $L^{-1}$ (Tomlin 2000) and 33.2 µg $L^{-1}$ (van Wijngaarden et al. 2005)], what is consistent with the stimulation of the evaluated endpoints for both cladoceran species.

On these grounds, a stimulus caused by the organic and bacterial enrichment of water samples and elutriates, particularly noticed during the rice culture, could be superimposed to their potential toxic effects, what, in turn, can represent confounding factors of bioassay responses.

The results of the present study seem to point out that the indicators of biological response should be chosen for their sensitivity and also for their ability to highlight the influence of different interacting factors.

## 5.5 Conclusions

The agricultural practices carried out during the rice growing season represented a risk to the nearby aquatic system, mainly elicited by the general degradation of its physico-chemical conditions, and also by the significant inhibition of microalgae growth when exposed to environmental samples. During the rest of the year, the whole system seemed to recover from agricultural aggressions, once before the beginning of a new rice culture the impacts previously determined were more reduced.

On the whole, the site L1 and L2 presented poorer quality. They showed an advanced trophic state sustained by high nutrient loads, Chl *a* content and bacterial densities, especially during the rice season. These conditions raise some concern, especially regarding the conservation of habitats within the protected wetland to which L1 belongs to.

Within the studied environmental matrices/compartments, sediment/soil elutriates, on one hand, were the most toxic samples for microalgae growth through the sampling sites and periods; while, on the other, they stimulated the life-history traits of daphnids. Though pesticides were only detected in elutriate samples from L2 and L3 during the rice culture, their mere concentrations were not apparently responsible for the toxicity revealed by algae. However, their stimulatory effect on daphnids could be associated to the surplus ingestion of bacteria and nutrient enriched suspended particles, which may mask the potential toxic effect of elutriates for them. Notwithstanding, the most sensitive trophic level was the phytoplankton.

Overall, the application of WET tests through the use of organisms belonging to different trophic niches proved to be a valuable tool to discern hazardous environmental samples, under a toxicity screening evaluation level. Anyway, future works should address more realistic scenarios to fulfil a broad toxicity profile that is lacking when confounding factors may dissimulate potential hazards and difficult the establishment of cause-effect relationships.

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# Chapter VI

*In situ* aquatic bioassessment of pesticide application on rice fields using a microalga and daphnids

# *In situ* aquatic bioassessment of pesticide application on rice fields using a microalga and daphnids

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## Abstract

This study assessed the effects of episodic contamination on a drainage canal adjacent to an area of intensive rice production (Coimbra, Portugal), where great amounts of herbicides and nutrients are used. Four monitoring periods were considered [before herbicide application (day -14), at first application day (day 0), 3, 5 and 6 days after that]. Each one consisted in three complementary evaluation lines: i) physico-chemical analyses, ii) whole effluent toxicity (WET) assays with *P. subcapitata*, iii) *in situ* bioassays to assess microalgae (*Pseudokirchneriella subcapitata*) growth, and the feeding rate and survival of autochthonous (*Daphnia longispina*) and standard (*Daphnia magna*) daphnids. Study sites were located upstream, in a protected wetland (L1), and downstream, in the vicinity of rice fields (L2). Along with the application of agrochemicals, there was a general decrease of water quality, especially in L2, due to nutrient and herbicide inputs. Herbicide peaks (on days 0, 5 and 6) in L2 water samples were recorded concomitantly or immediately after their application. The maximum concentrations measured for propanil were detected on day 0; for penoxsulam, MCPA, and 3,4-DCA on day 5; bentazone and MCPA on day 6. Regarding the *in situ* bioassessment, the algae growth decrease from day 0 onwards in L1, whilst in L2 its inhibition was generally coherent with the decline of water quality. Apparently, WET tests indicated that the limitation of nutrients could be affecting algae growth in L1, but it did not seem to be the sole factor in L2, hence conclusions from WET tests should be cautious. The feeding depression of daphnids occurred on days 0 and 5 for *D. longispina* and only on day 0 for *D. magna*, while significant reductions on survival were restricted to day 0 for both species. The impairments occurring on day 0 were linked to a potential increased toxicity driven by the surplus ingestion of particle-bound herbicides due to runoff events. Overall, the feeding rate of daphnids provided an earlier indication of toxic impairments, though it is prompted the use of complementary endpoints and trophic levels in order to improve the understanding of cumulative effects of herbicide pulses.

**Key-words:** *in situ* bioassays, herbicides, *Pseudokirchneriella subcapitata*, *Daphnia longispina*, *Daphnia magna*, growth, feeding inhibition, survival

## 6.1 Introduction

Rice is one of the largest produced cereals in the world (Nguyen 2002) that is cultivated under submerged conditions and requires the application of great amounts of pesticides, specially herbicides (Kuster et al. 2008, Comoretto et al. 2008). In Europe, rice is often cropped within protected areas presenting high natural value (Tarazona and Sánchez 2006), though it has been frequently detected great quantities of herbicides (at μg $L^{-1}$ level) in irrigation/drainage canals and in adjacent waterbodies (*e.g.*, Santos et al. 2000, Konstantinou et al. 2006, Barata et al. 2007, Faria et al. 2007, Comoretto et al. 2008, Kuster et al. 2008).

Indeed, the contamination of those surface waters assumes a typical episodic profile mainly due to spray drift, aerial deposition and runoff of pesticides during their pulsed application (Boxall et al. 2002, Dabrowski et al. 2005). Consequently, non-target aquatic wildlife may be exposed to short-term peaks of herbicides, hence strengthening the need for evaluating intermittent exposure scenarios, as long as they may produce stronger cumulative impairments than would continuous exposures do (Tucker and Burton 1999, Boxall et al. 2002). Thereby, when it comes to perform site-specific assessments, the application of low tier approaches addressing the use of laboratorial tests under standard, continuous and unrealistic conditions, though useful, they may overlook and underestimate the actual risks occurring under field-relevant fluctuating exposures (Chappie and Burton 1997, Tucker and Burton 1999, Boxall et al. 2002).

*In situ* bioassays, however, are usually recommended for a higher-tier assessment level since they integrate the effects of contaminants and environmental variables on a spatial and temporal scale, hence providing more ecologically relevant estimations than laboratorial tests (Chappie and Burton 1997, Tucker and Burton 1999). Also, they hamper the influence of artifacts associated with laboratorial exposures such as the collection, storage and handling of samples, while allowing the control of the organisms' inherent conditions (*e.g.*, physiological condition, age, size) (Chappie and Burton 1997). Specifically, *in situ* bioassays have been considered a helpful tool for assessing non-point source of contaminants (*e.g.*, Chappie and Burton 1997, Tucker and Burton 1999, Schulz 2003). In particular, caged organisms were already deployed in aquatic systems nearby agricultural areas to evaluate the effects generated by pulses of single or multiple pesticides (*e.g.*, Schulz 2003, Phillips et al. 2004, Faria et al. 2007, Domingues et al. 2008).

Along with the prompted use of *in situ* bioassays to assess ecological risks several organisms belonging to different trophic levels had been used, namely microalgae (*e.g.*, Twist et al. 1997, Moreira et al. 2004a, 2004b), macrophytes (*e.g.*, Graça et al. 2002), cladocerans (*e.g.*, Pereira et al. 1999, McWilliam and Baird 2002a, Phillips et al. 2004, Barata et al. 2007, Damásio et al. 2008), amphipods (*e.g.*, Chappie and Burton 1997, Schulz 2003), chironomids (*e.g.*, Castro et al. 2003, Faria et al. 2007, Domingues et al. 2008) and fish (Castro et al. 2004).

Among those groups, microalgae and daphnids are widely recommended for ecotoxicological testing, being both considered sensitive test organisms to herbicide effects (EC 2002). As a matter of fact, they belong to two basic trophic levels – producers and primary consumers, respectively – that sustain and allow energy transfer along freshwater trophic chains (Källqvist and Romstad 1994, Hanazato 2001, EC 2002). As such, any impairments occurring on algae and daphnid fitness due to chemical exposures may constrain the maintenance of natural populations, what in turn may induce bottom-up and top-down deleterious effects on freshwater ecosystems (Källqvist and Romstad 1994, Allen et al. 1995), compromising their equilibrium and sustainability. Under this context, the evaluation of sensitive sub-lethal endpoints at the individual-level like growth and feeding activity may provide a closer understanding of potential consequences at the population-, community-, and ecosystem-level (Barata et al. 2007). In particular, the immobilisation of *Pseudokirchneriella subcapitata* in calcium alginate beads for *in situ* assessments had provided successful growth performances, while mitigating cell loss by bead degradation, water flow or predation (*e.g.*, Moreira–Santos et al. 2004b, Moreira et al. 2006). In turn, the filter-feeding activity of daphnids is an ecologically relevant endpoint, which may be strongly altered under low toxic exposures, thereby providing an earlier indication of impairments than survival rates (Allen et al. 1995, McWilliam and Baird 2002b, Barata et al. 2007).

The present work aimed to study in what extent the pulsed application of herbicides on a rice cropping area (Montemor-o-Velho, Centre of Portugal) impacted the quality of a main drainage canal, which crosses a protected wetland upstream and rice fields downstream. For that, four assessment moments were essentially considered: before the beginning of agricultural practices (day -14), at the first herbicide application (day 0), 3 (day 3), 5 (day 5) and 6 (day 6) days afterwards. The experimental design was established along a temporal scale in order to gauge potential intermittent threats and system recovery, thereby increasing field-exposure realism. Each assessment period involved different concomitant analyses: i) the monitoring of water physico-chemical parameters, ii) the deployment of *in situ* bioassays to tease out changes on *P. subcapitata* growth, and on the feeding rate and survival of autochthonous and standard cladocerans (*Daphnia longispina* and *Daphnia magna*, respectively), and iii) the development of whole effluent toxicity (WET) tests with *P. subcapitata* (to overcome interaction with possible confounding factors like deficient light, temperature or nutrient content). Unlike other previous studies, this work allowed the simultaneous use of two trophic levels to assess diffuse agricultural pollution. Additionally, the sensitiveness of species and measured endpoints to evaluate episodic contamination scenarios was compared and discussed. At last, the gathered information from *in situ* bioassays with algae was integrated with that of WET assays conducted in the laboratory to boost data interpretation.

## 6.2 Material and Methods

### 6.2.1 Study area, sampling sites and herbicide treatment

The study sites were integrated within an area intensively used for agricultural activities, being located in the Lower Mondego river Valley (centre of Portugal, near Coimbra) (40º 2' N, 8º 43' W). It is one of the most important Portuguese regions of rice production, comprising 15000 ha of agricultural land that is used for corn production, as well, but in much lower extent. In the proximity of this area there is a wetland – Paul do Taipal - that was indeed used for rice culture until the 70 decade; nevertheless, in 1999 it was classified, by national regulation (Law by Decree no. 384-B/99, 23.09.1999), as a special protection zone for birds, and hence integrated in the Natura 2000 Network (EEC 1979, EEC 1992, ICN 2008) (code no. PTZPE0040). Furthermore, in 2001, it was integrated on the Ramsar List of Wetlands of International Importance (Ramsar site no. 1107).

The overall hydro-agricultural scheme of the Lower Mondego river Valley is constituted by a widespread irrigation and drainage network (Lima and Lima 2002), being the water flux in the canals/ditches controlled by dams constructed in downstream strategic points. As such, the *in situ* study was conducted in an irrigation/drainage canal that is about 5 km long, 6 – 7 m wide and, during the study period, it presented a water depth varying between 1 - 2.5 m. The canal crosses the protected wetland located upstream and downstream, the agricultural fields (fig. VI.1). Two study sites were chosen, one (L1) was located in a canal reach within the protected wetland, and the other (L2) was in a canal reach surrounded by rice fields (fig. VI.1). The water flow was almost residual (≤ 0.3 m $s^{-1}$) through the *in situ* assessment period, because downstream dams were closed. The great percentage of silt/clay content characterising the particle-size distribution of sediments (7.3 - 26.3%) and paddy soil (43.1 - 53.3%) favours the high input of suspended solids that gave a turbid appearance to the flowing water. The main difference between L1 and L2 is that the former presents a dense cover of macrophytes (mainly characterised by *Typha latifolia*, *Scirpus lacustris* and *Phragmites australis*) along the canal banks, thereby enhancing the shadow area relatively to that observed for L2. Moreover, the water in L1 was almost standing due to the construction of a partial barrier only sometimes allowing the communication with downstream water of the canal.

The application of agrochemicals in that area and, more specifically for the rice culture, occurs mainly during the end of April up to June, and it is conducted either through terrestrial or aerial spraying. The commonly applied fertiliser was ammonium sulphate. Among the applied pesticides, herbicides are the ones mostly used, such as Viper® (2.4 g penoxsulam $L^{-1}$), Stam Novel Flo 480® (480 g propanil $L^{-1}$), Basagran® (480 g bentazone-Na $L^{-1}$), Quitt® (400 g bentazone $L^{-1}$ + 60 g MCPA $L^{-1}$), and Facet® (250 g quinclorac $L^{-1}$). Whenever the pests can not be controlled through the dryness of paddy fields farmers make confined applications of the insecticides Quirlan® (24% chlorphenvinfos $L^{-1}$; SL) or Decis® (25 g deltamethrin $L^{-1}$; EC). However, as far as authors are aware,

no insecticides were applied during the rice culture in the year where the sampling was conducted (personal communication of farmers).

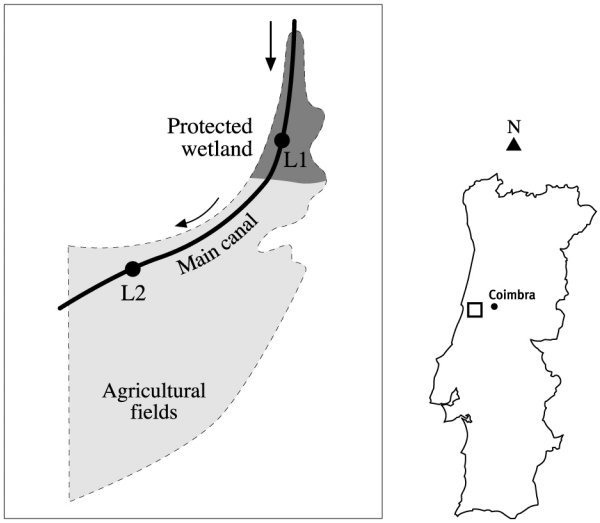


Figure VI.1 - Schematic representation of the study area and sites. The shaded area in dark grey represents the protected wetland, while the light grey one roughly indicates the agricultural field area.

### 6.2.2 Test organisms and rearing conditions

*P. subcapitata* Korshikov (Hindak) was maintained in unialgal batch cultures with 100mL Woods Hole MBL medium (referred as MBL), at 20 ± 2ºC and $16^{L}$:$8^{D}$h (light provided by cool-white fluorescent lamps). New cultures were initiated through algae harvest from cultures at the exponential growth phase (*i.e.* 5 - 7 days-old) following its inoculation into fresh medium.

Monoclonal bulk cultures of *D. longispina* [clone EM7, *sensu* Antunes et al. (2003), isolated from a population collected in Lake Vela, and maintained for several generations in the laboratory] and *D. magna* [clone A, *sensu* Baird et al. (1989a)] were reared in ASTM (ASTM 1980) enriched with a standard organic additive (*Ascophylum nudosum* seaweed extract; Baird et al. 1989b), under 20 ± 2 °C and a $16^{L}$:$8^{D}$ photoperiod. Cultures were renewed and fed (with *P. subcapitata* at a rate of 1.50 and 3.00 x $10^{5}$ cells $mL^{-1}$/*Daphnia* for *D. longispina* and *D. magna*, respectively) every other day.

### 6.2.3. *In situ* experimental setup and assessment

The *in situ* assessment was performed in 2007 from mid of May up to the beginning of June. As already said, the *in situ* bioassays were deployed along a time-scale profile, which was defined according to the application pulses of herbicides: i) before overall herbicide application (preliminary test at day -14), ii) at the application of Viper (day 0), iii) 3 or 5 days after the initial application of Viper (days 3 or 5), iv) 6 days after the application of Viper (day 6) (table VI.1).

Table VI.1 - Schematic diagram of the time-scale deployment of *in situ* bioassays (AGR – algae growth rate, DFR – daphnid feeding rate, DS – daphnid survival) relatively to the application pulses of herbicides (shadowed columns).

| Test days | -14 | 0 | 3 | 5 | 6 |
|---|---|---|---|---|---|
| Type of bioassays deployed | AGR | AGR | AGR | DFR | AGR |
| | DFR | DFR | DS | | DFR |
| | DS | DS | | | DS |

Each set of tests involved the: a) physico-chemical characterisation of the aquatic system, immediately before the deployment of testing structures, b) development of microalgal growth bioassays (AGR), c) development of bioassays with daphnids to ascertain their potential feeding depression (DFR) and survival (DS). As long as it was possible, the bioassays were performed according to procedures outlined in the respective standard protocols [for algae growth (OECD 2002) and daphnid survival measured as immobilisation (OECD 2004)].

*a. Physical and chemical characterisation*

The parameters evaluated *in situ* were pH (pH 330 from WTW), temperature, conductivity (LF 330 from WTW), concentration of dissolved oxygen ([$O_2$], Oxi 330 from WTW) and Secchi disc transparency. Additionally, sub-surface water samples were collected in each site and simultaneously filtered through a 55-µm filter into glass vessels. They were stored at ≈ 4ºC in darkness. In the laboratory, those water samples were processed to determine the concentration of Chl *a* ([Chl *a*]), the total suspended solids (TSS), and the nutrient content based on the concentration of nitrates ($NO_3^-$-N), nitrites ($NO_2^-$-N), un-ionised ammonia ($NH_4^+$ measured as $NH_3$-N), phosphates ($PO_4^{3-}$) and sulphates ($SO_4^{2-}$) (A.P.H.A. 1995).

Furthermore, field water samples from L1 and L2 were screened for the presence of herbicide active ingredients (a.i.), which were likely to reach the aquatic environment during their intermittent application. They were, penoxsulam, quinclorac, bentazone, MCPA, propanil and its major metabolite 3,4-dichloroaniline (3,4-DCA) (Santos et al. 1998). The main characteristics of these compounds are presented on table VI.2. To meet this purpose, samples were filtered through a 0.45-µm nylon membrane (Whatman) and submitted to solid phase extraction (SPE). For penoxsulam analysis, 1 L of sample was passed through a 500 mg Sepra SDB-L (95 µm, 255A) cartridge (purchased from Phenomenex). The cartridge was previously activated by flushing with 4 mL of methanol followed by 4 mL of water. After loading the sample into SPE cartridge, it was washed with methanol:water (5:95) and dried under vacuum for 30 min. Penoxsulam was eluted with 5 mL of acetonitrile with 0.1% acetic acid. Regarding the other four herbicides and 3,4-DCA, 1 L of sample,

acidified to pH 3 with phosphoric acid, was passed through a 500 mg C18 cartridge (purchased from Phenomenex), previously activated by flushing with 3 mL of MeOH, 3 mL of milli-Q water and 3 mL of milli-Q water at pH 3. Extraction cartridges were then washed with 5 mL of water and dried under vacuum for 30 min. Elution was performed with 5 mL of MeOH without open the vacuum. The eluates of all six herbicides and 3,4-DCA were evaporated to dryness under a gentle stream of nitrogen and the residues were then redissolved in 250 µL of mobile phase.

The analytical instrumentation included an HPLC Jasco model with a Rheodyne 7125 injector and a loop size of 50 µL coupled to an UV detector UV Chrom-A-Scope (BarSpec). For penoxsulam determination the analytical column was a Supelcosil LC-8 (150 x 4.6 m; 5 µm, 120 A), with a guard column of the same material. The mobile phase selected consisted of water acidified with 0.01% of acetic acid and 40% of acetonitrile, at a flow rate of 1 mL $min^{-1}$. For quinclorac, bentazone, MCPA, propanil and 3,4-DCA, the analytical column used was a Luna C-18 (250 x 4.6 m; 5 µm, 100 A), with a guard column of the same material. The mobile phase selected consisted of water acidified to pH 3 with phosphoric acid and 40% of acetonitrile at a flow rate of 1 mL $min^{-1}$. The temperature of the analytical column was set to 30ºC in both cases.

The limit of quantification (LOQ) of the a.i.s in samples, after pre-concentration, calculated as ten times the signal-to-noise, ranged from 0.05 µg/L (for quinclorac) to 0.07 µg $L^{-1}$ (for propanil and penoxsulam). The mean recovery obtained for the spiked level tested (0.23 µg $L^{-1}$ for penoxsulam and 1.0 µg $L^{-1}$ for the other pesticides) ranged from 75 $\pm$ 3% (for MCPA) to 101 $\pm$ 4% (for penoxsulam).

*b. Immobilisation of microalgae in beads*

*P. subcapitata* was immobilised in calcium alginate beads according to methods outlined in Moreira dos Santos et al. (2002) and Moreira-Santos et al. (2004a, 2004b). First of all, a 1.3% (w/v) sodium alginate solution (Fluka BioChemika 71238, CAS nº: 9005-38-3) was prepared with sterilised distilled water (15' at 120ºC) previously warmed up. After centrifuging an aliquot of algal culture (5' at 3500 rpm) at the exponential growth phase, it was ressuspended in MBL and, afterwards, < 1 mL of this concentrated algal suspension was added to the alginate solution as to obtain an alginate-cell suspension with *ca.* $10^6$ cells $mL^{-1}$ initial cell density. Following this, a 2% (w/v) solution of $CaCl_2$ (Merck 1.02382, CAS no. 10043-52-4) was prepared to form the beads at a rate of one drop of the alginate-algae suspension *per* second through a sterilised needle coupled to a 20-mL syringe.

Beads were stirred in the $CaCl_2$ solution during approximately 45 min., in order to achieve gel hardening. After that they were washed in distilled water and stored in darkness, for a maximum of 15 days, in 20-times diluted MBL solution at 4ºC.

Table VI.2 - General information and physico-chemical characteristics of the analysed herbicides and the by-product 3,4-DCA. Most of the data was based on Tomlin (2000), except when indicated by the superscript numbers.

| | Penoxsulam | Quinclorac | Bentazone | MCPA | Propanil | 3,4-DCA |
|---|---|---|---|---|---|---|
| Application rates | 2 L $ha^{-1}$ | 2.5-3 L $ha^{-1}$ | 3-4 L $ha^{-1}$ | 3-3.5 L $ha^{-1}$ | 7.5-14 L $ha^{-1}$ | - |
| Formulated product | Viper® | Facet® | Basagran® | Quitt® | Stam Novel Flo 480® | - |
| Company | Dow AgroSciences | Basf | Basf | Basf | Cequisa | - |
| Formulation type | EC | SC | S | S | SC | - |
| Chemical group | Triazopyrimidine sulfonamide | Quinolinecarboxylic acid | Benzothiazinone | Cholorophenoxy acid | Anilide | Aniline |
| Molecular weight (g $mol^{-1}$) | 483 [1] | 242 | 240.3 | 200.6 | 218.1 | 162 [10] |
| Solubility (mg $L^{-1}$) | 410 (pH 7)[2] | 0.065 (20ºC)[5] | 570 (20ºC, pH 7) | 734 (25ºC) | 130 (20ºC) | 580 (20ºC)[10] |
| Vapour pressure (mPa) | $9.5x10^{-11}$ (25ºC)[1] | <0.01 (20ºC) | 0.17 (20ºC) | $2.3x10^{-2}$ (20ºC) | 0.02 (20ºC) | 184 (20ºC)[10] |
| Log $K_{ow}$ | 0.602 - 8 (pH 7)[3] | -1.15 (pH 7) | -0.46 (pH 7) | 0.46 (pH 5) | 3.3 (20ºC) | 2.69 [9] |
| $K_{oc}$ | 104 [4] | 50 | 42 | 7 [4] | 149 [7] | 338.6 [10] |
| Half-lives (days) | | | | | | |
| soil | 34-118 [3] | 450 [4] | 12 to 45 | 25 [4] | 1 [7] | 1000 [11] |
| water | 12-38 [3] | - | 80 [4] | 13.5 [4] | 0.5-1[7] | stable[11] |
| photolysis | 1.5-14 (in water) [3] | - | 4 (pH 7)[4] | 0.05 [4] | 0.5 (in water, pH 7)[4] | 18 [10] |
| Ecotoxicity | | | | | | |
| Microalgae | | | | | | |
| 72-h $EC_{50}$ (mg $L^{-1}$) | 0.47 [4] (*Anabaena flos-aquae*) | 6.53 [4] (*Pseudokirchneriella subcapitata*) | 47.3 | 21.96 [6] (*Chlorella pyrenoidosa*) | 0.031 (96-h $EC_{50}$)[8] (*Pseudokirchneriella subcapitata*) | 3.2 (96-h $EC_{50}$)[12] |
| *Daphnia* sp. | | | | | | |
| 48-h $EC_{50}$ (mg $L^{-1}$) | 98.3 [4] | 29.8 [4] | 125 | 190 [4] | 3.55 - 6.72[9] | 0.23 [11] |

EC -emulsifiable concentrate, S - solution, SC - sprayable concentrate. [1] Jabusch and Tjeerdema 2008, [2] Roberts et al. 2003, [3] U.S.EPA 2007, [4] FOOTPRINT 2008, [5] Marchesan et al. 2007, [6] Ma et al. 2001, [7] Konstantinou et al. 2006, [8] Pereira et al. (in press), [9] Pereira et al. 2007, [10] Gonzáles-Pradas et al. 2005, [11] EC 2006, [12] Mayer et al. 1998.

The beads hence obtained presented a mean diameter of (2.9 ± 0.018 mm). In order to proceed with cell counting after the end of the test, 3 beads per replicate were disaggregated in 1 mL of trisodium citrate solution [3% (w/v); Riedel-de Häen 25116, CAS no. 6132-04-3] upon smooth shaking. The cell density of each 3 bead-replicate was determined by cell counting on a microscope Olympus CKX41 using a Neubauer chamber.

*c. Microalgal growth bioassays*

The constructed apparatus for microalgae bioassays was adapted from that developed and described in detail by Moreira dos Santos et al. (2002) and Moreira-Santos et al. (2004a, 2004b). It was a simple, cost-effective, and efficient system basically constituted by a plate for bead exposure [PBE *sensu* Moreira dos Santos (2002)] and an outer recipient responsible for the protection and reduction of detritus accumulation on the PBE structure. The PBE consisted on a 24-well microplate, in which the top and removed bottom of 4 wells were closed with a 55-µm nylon mesh. The outer recipient for the control (no contact with water system) and test (direct contact with water system)

systems was different. The control outer recipient consisted in a transparent polyethylene bag filled with MBL medium that was sealed after introducing the PBE. This structure was then placed into a 5-mm net bag that guaranteed further resistance of the whole control system. In turn, the outer recipient of the test system was made of a polyethylene 5-L bottle in which transversal holes were cut and wrapped with a 0.5-mm nylon mesh. Non-toxic white thermal glue was used during the construction of the structures (Pereira et al. 1999), which were left in dechlorinated tap water for 24h before their use.

The control systems (with enclosed MBL) and the PBE structures for the test systems were prepared in the laboratory prior to their transport to the field (in the storage medium of beads, at 4ºC in darkness). Once in the field, they were introduced in the respective outer recipient, before their *in situ* deployment. Meanwhile, nine beads (*i.e.*, 3 replicates of 3 beads) were conserved in Lugol's solution to allow the determination of the effective initial cell density. In each site were deployed four replicates of the control and test systems. On a whole, one replicate consisted in an outer recipient, a PBE structure bearing 4 sub-replicates (wells), which contained 3 beads each. Each replicated system was tied up to a main rope fixed to both margins of the canal, in a way that allowed both their submersion and the penetration of light. After a 9-day exposure (*c.f.*, Moreira-Santos et al. 2004a) the *in situ* systems were removed and the beads were conserved in Lugol's solution until cell counting in the laboratory (on a microscope Olympus CKX41 using a Neubauer chamber), to ascertain microalgae growth. Two validity criteria were taken into account, according to the established by the OECD guideline (2002) for algae growth assay: (i) the cell density in controls should increase by a 16-fold and (ii) the coefficient of variation of the average growth rate should be ≤20%.

*d. Bioassays with daphnids*

The apparatus used for daphnid tests was adapted from that used and validated by Pereira et al. (1999). Hence, the constructed system involved an outer 1-cm mesh rectangular structure with approximate dimensions of 50x10x5 cm, within which 10 chambers (60-mL polyethylene flasks with a lid) were fixed with a plastic wire. Five replicate control chambers were completely closed and filled with ASTM medium. The other five replicate chambers were designed for direct contact with field water (*i.e.*, test chambers) and hence, two lateral squared windows (2 cm side length) and one on the lid (2 cm diameter) were opened and covered with a 55-µm nylon mesh that was fixed with the same aforementioned glue. The net used prevented the entrance of major organisms and detritus, while it allowed the continuous flow of field water.

As such, it was deployed one *in situ* system *per* daphnid species (*i.e.* for *D. longispina* and *D. magna*) and test type (*i.e.*, for feeding inhibition and survival assays), hence totalling four complete

structures *per* site. Daphnids were transported to the field in 1 L flasks containing 800 mL of ASTM. For the feeding inhibition bioassay were assigned 12 individuals of *D. longispina* (3- to 4-day-old) and 7 of *D. magna* (4- to 5-day-old) to enhance the chance of having at least 10 and 5 organisms, respectively, at the end of the test for the feeding post-exposure period (see below). For the survival bioassay, however, 5 newborn neonates (< 24-h) were assigned to each replicated control and test chambers. The *in situ* structures were submersed lid downward using weights attached to the outer net structure that maintained the chamber position, hence avoiding a great accumulation of suspended solids. Similarly to microalgae, these structures were fastened to a main rope that was fixed to the margins.

Feeding inhibition bioassays were set up having in consideration the methodology followed by Allen et al. (1995) and McWilliam and Baird (2002a, 2002b). This assay consisted in a 24-h *in situ* exposure period plus a 4-h *ex situ* post-exposure feeding period. After the 24-h *in situ* exposure, the chambers were retrieved and checked for dead individuals. The daphnids alive (optimally 10 for *D. longispina* and 5 for *D. magna*) were then immediately transferred to 60-mL polypropylene vessels containing 50 mL of ASTM with *P. subcapitata* at a concentration of 1.50 and 3.00 x $10^5$ cells.$mL^{-1}$/*Daphnia* for *D. longispina* and *D. magna*, respectively. Animals were left to feed for 4-h in darkness (*c.f.*, McWilliam and Baird 2002a) at 20±2ºC, what corresponded to the post-exposure feeding period. Three replicates without animals were used to measure initial algal cell densities. At the end of the 4-h period, the animals were removed and an aliquot of the homogenised algal suspension was retrieved from each replicate to be treated with Lugol's solution. Afterwards, algal cells were counted (on a microscope Olympus CKX41 using a Neubauer chamber) for the subsequent determination of daphnids' feeding rates. The period of exposure of the survival bioassay was 48-h (OECD 2004). At the end of the assay, the respective chambers were removed and the number of recovered and alive organisms was counted.

For this part of the experimental work no WET tests were conducted, since the main environmental factors that could affect *Daphnia* sp. feeding and survival (TSS, temperature, water flow) did not assume values able to decrease their biological responses (according to the studies of Tucker and Burton 1999, McWilliam and Baird 2002a, 2002b). Moreover, different studies have found that laboratorial assays underestimate or give similar estimations of actual effects occurring under realistic *in situ* scenarios (*e.g.,* Tucker and Burton 1999, Graça et al. 2002, Schulz 2003, Phillips et al. 2004). Therefore, and having in mind the proposed goals, we found unnecessary the performance of WET tests with daphnids, as they would not improve that much study reliability or data interpretation.

### 6.2.4 Laboratory WET tests with microalgae

The WET tests were simultaneously conducted with the *in situ* microalgae assays, as to improve the interpretation of the latter since some confusing environmental factors could be dismissed under laboratory conditions (*e.g.*, light intensity due to macrophyte cover or high quantity of suspended matter, temperature variation and nutrient limitation) (Mayer et al. 1998, Moreira-Santos et al. 2004b). Green algae 96h-assays were then carried out with beads of *P. subcapitata* by following the procedures outlined in the U.S.EPA (2002) and OECD (2002) guidelines. Site waters from L1 and L2 were pre-filtered (through a GF/C filter). Three replicates containing four beads (with initial cell density *ca.* $10^6$cells.mL$^{-1}$) each were used per treatment. Two treatments were considered: (i) 100% plain water (L1 and L2), (ii) site water enriched with nutrients (L1+N and L2+N) added in the same concentrations as recommended for MBL medium. The tests were conducted under constant agitation (≈ 100 rpm in an orbital shaker) in the same conditions of algal cultures, with a light intensity ranging between 90.98 and 108.16 µmol.s$^{-1}$.m$^{-2}$ (or 4665.64 and 5546.66 lux). At the end of the test, the beads were preserved with Lugol's solution. Cell density (counting of cells on a microscope Olympus CKX41 using a Neubauer chamber) was the biomass parameter used for the calculation of the microalgae growth rate.

### 6.2.5 Data analysis

All data generated from the *in situ* and laboratory bioassays were expressed as percentage of the respective controls, in order to minimise differences associated to the experimental apparatus, intrinsic condition of the test organisms (Chappie and Burton 1997) and/or environmental factors. The average microalgae growth was determined according to OECD guideline (2002) from the initial and final logarithmic cell densities, for the *in situ* and WET bioassays. For the *in situ* feeding depression and survival assays with daphnids was calculated the average percentage of recovered daphnids (dead or alive) from the chambers, as a way to ascertain the validity of the conducted approach. The average percentage of surviving animals after the 48-h exposure period was determined as well. Whilst the average feeding rate was estimated from a simplified version of the Gauld's equation according to Allen et al. (1995) and Orchard et al. (2002) that was applied to each replicate:

$$F = [V \times (C_i - C_f)] / (n \times t),$$

where $F$ is the feeding rate in cells/*Daphnia*/h, $V$ is the volume of ASTM medium (mL), $C_i$ is the initial algal density, $C_f$ is the final algal density, $n$ is the number of daphnids and $t$ is the exposure period (h) which was 4h in the present study.

For the *in situ* and WET assays, a two-way analysis of variance (two-way ANOVA) (Zar 1996) was applied to assess the significance of the effects of testing periods and sites, as well as their interaction on the microalgae growth rate (three replicates), survival and feeding rate (three replicates) of both daphnid species. Whenever a significant interaction between those factors was detected, a one-way ANOVA was used for each factor followed by the Tuckey multiple comparison test, so as to identify significant differences between the treatments within the other factor (Zar 1996). Furthermore, algae growth rates in *in situ* and WET assays were compared by the Student's *t*-test, within each site and testing period. Also, for the WET assays with algae, a Student's *t*-test was carried out within each site and testing period to compare the effect of treatments with plain water and nutrient enriched site water. Statistically significant differences were indicated for $P \leq 0.05$.

## 6.3 Results

Table VI.3 shows the physical and chemical parameters measured in in both sites, at the moment of deployment of the *in situ* bioassays. The pH and conductivity values were nearly constant along the bioassessment period, varying between 7.4 - 7.8 and 422 - 563 µS $cm^{-1}$, respectively. It was observed an increasing trend for the temperature along the testing periods, for both sites. That trend was followed by the decreasing of $[O_2]$ from day 0 onwards, although the lowest values were always recorded in L1 (3.2 - 8.9 mg $L^{-1}$). The highest transparency was recorded for day -14 at L1, which was then reduced in the subsequent testing days, maintaining similar values for both sites (0.2 – 0.3 cm). However, the highest records for total suspended solids were determined at the day 0 of test, for both sites, what was congruent to the rainfalls simultaneously occurring. The [Chl a] was generally higher in L1 (10.3 - 22.7 µg $L^{-1}$) than in L2 (3.9 - 15.5 µg $L^{-1}$). The lowest values were determined in L2 at the days of pesticide application (days 0 and 5) (5.9 and 3.9 µg $L^{-1}$, respectively). Relatively to the nutrients, except for $NO_2^-$, their aquatic concentrations had generally increased along the testing periods in L2, ranging between 0.40 - 0.70 mg $NH_3$-N $L^{-1}$, 0.90 - 1.80 mg $NO_3^-$-N $L^{-1}$, 0.001 - 1.00 mg $NO_2^-$-N $L^{-1}$, 0.06 - 0.59 mg $PO_4^{3-}$ $L^{-1}$ and 19 - 29 mg $SO_4^{2-}$ $L^{-1}$. On the other hand, in L1, the broad pattern indicated higher nutrient concentrations at day -14, which reduced at days 0 and 3 and, in some situations, rose again at days 5 and 6. They were between 0.23 - 0.74 mg $NH_3$-N $L^{-1}$, 0.70 - 2.90 mg $NO_3^-$-N $L^{-1}$, 0.00 - 4.00 mg $NO_2^-$-N $L^{-1}$, 0.12 - 0.32 mg $PO_4^{3-}$ $L^{-1}$ and 15 - 31 mg $SO_4^{2-}$ $L^{-1}$.

In what concerns the quantification of pesticides, it was noticeable a discrepancy among L1 and L2 contamination profiles, as it was expected. Thereby, the highest pesticide concentrations were determined in L2 on days 0, 3, 5 and 6. On the first application day (day 0) was observed a peak

concentration of propanil (2.4 µg $L^{-1}$), whilst on day 3, MCPA was the herbicide mostly quantified (2.5 µg $L^{-1}$).

Table VI.3 - Physico-chemical parameters monitored at the deployment days of the *in situ* bioassays, in local 1 (L1) and 2 (L2). The shadowed columns indicate days of pulsed applications of herbicides.

| Days of test | -14 | | 0 | | 3 | | 5 | | 6 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sites | L1 | L2 | L1 | L2 | L1 | L2 | L1 | L2 | L1 | L2 |
| pH | 7.7 | 7.8 | 7.4 | 7.6 | 7.5 | 7.6 | 7.5 | 7.4 | 7.5 | 7.5 |
| Conductivity (µS $cm^{-1}$) | 422 | 492 | 508 | 475 | 561 | 563 | 535 | 479 | 503 | 530 |
| [$O_2$] (mg $L^{-1}$) | 8.9 | 12.6 | 6.4 | 7.8 | 4.4 | 6.1 | 4.9 | 5.2 | 3.2 | 4.6 |
| Temperature (ºC) | 16.0 | 16.9 | 18.9 | 19.7 | 19.5 | 19.5 | 21.8 | 22.5 | 22.4 | 22.3 |
| Transparency (m) | 0.6 | 0.3 | 0.3 | 0.3 | 0.2 | 0.2 | 0.3 | 0.2 | 0.3 | 0.2 |
| TSS (mg $L^{-1}$) | 0.89 | 0.94 | 0.96 | 0.97 | 0.84 | 0.81 | 0.82 | 0.76 | 0.81 | 0.77 |
| [Chl *a*] (µg $L^{-1}$) | 10.7 | 9.8 | 22.7 | 5.9 | 11.9 | 9.10 | 16.3 | 3.9 | 10.3 | 15.5 |
| [Nutrients] (mg $L^{-1}$) | | | | | | | | | | |
| $NH_3$-N | 0.23 | 0.40 | 0.45 | 0.49 | 0.71 | 0.66 | 0.43 | 0.66 | 0.74 | 0.70 |
| $NO_3^-$-N | 2.90 | 0.90 | 0.70 | 1.80 | 0.80 | 1.40 | 1.00 | 1.50 | 0.90 | 1.40 |
| $NO_2^-$-N | 4.00 | 1.00 | 2.00 | 0.03 | 0.00 | 0.00 | 0.02 | 0.03 | 0.01 | 0.02 |
| $PO_4^{3-}$ | 0.16 | 0.06 | 0.14 | 0.25 | 0.17 | 0.35 | 0.32 | 0.59 | 0.12 | 0.36 |
| $SO_4^{2-}$ | 28 | 19 | 15 | 20 | 16 | 20 | 31 | 26 | 24 | 29 |
| [Pesticides] (µg $L^{-1}$) | | | | | | | | | | |
| Penoxsulam | bql | bql | 0.096 | 0.31 | bql | 0.099 | 0.084 | 2.3 | 0.0 | 0.86 |
| Quinclorac | bql | bql | bql | 0.18 | bql | 0.081 | 0.056 | 0.088 | bql | 0.053 |
| Bentazone | bql | bql | bql | 0.84 | 0.11 | 0.52 | 0.089 | 0.74 | bql | 1.1 |
| MCPA | bql | bql | bql | 0.37 | 0.20 | 2.5 | bql | 3.5 | bql | 3.0 |
| Propanil | bql | bql | bql | 2.4 | bql | 0.74 | bql | 0.62 | bql | 0.23 |
| 3,4-DCA | bql | bql | bql | bql | bql | bql | bql | 1.3 | bql | 0.50 |

[$O_2$] – concentration of dissolved oxygen, TSS – total suspended solids, [Chl a] – concentration of chlorophyll *a*, [Nutrients] – concentration of nutrients, [Pesticides] – concentration of pesticides, 3,4-DCA – 3,4-dichloroaniline, bql – below quantification limit.

On day 5 were broadly determined the highest concentrations of pesticides in L2 site water, since there was another input of MCPA (3.5 µg $L^{-1}$), in tandem with the detected peak concentrations of penoxsulam (2.3 µg $L^{-1}$), quinclorac (0.88 µg $L^{-1}$), bentazone (0.74 µg $L^{-1}$) and 3,4-DCA (1.3 µg $L^{-1}$), being the latter likely resulting from the degradation of propanil (0.62 µg $L^{-1}$). On day 6, MCPA was still present in higher concentrations (3.0 µg $L^{-1}$), together with bentazone (1.1 µg $L^{-1}$) and penoxsulam (0.86 µg $L^{-1}$), while quinclorac (0.053 µg $L^{-1}$), propanil (0.23 µg $L^{-1}$) and 3,4-DCA (0.50 µg $L^{-1}$) concentrations were decreasing.

The outcome of microalgae *in situ* and WET bioassays fulfilled the validity criteria defined on the OECD (2002) guidelines. The highest growth rates of *P. subcapitata* were recorded during the preliminary *in situ* assay for both sites (fig. VI.2). However, within each site, a significant reduction of microalgae growth was determined across the subsequent testing periods simultaneously to the

application of pesticides (fig. VI.2, table VI.4). While in L1 the significant impairment of algae growth occurred on days 0, 3 and 6, in L2 it was restricted to the bioassays deployed on days 3 and 6.

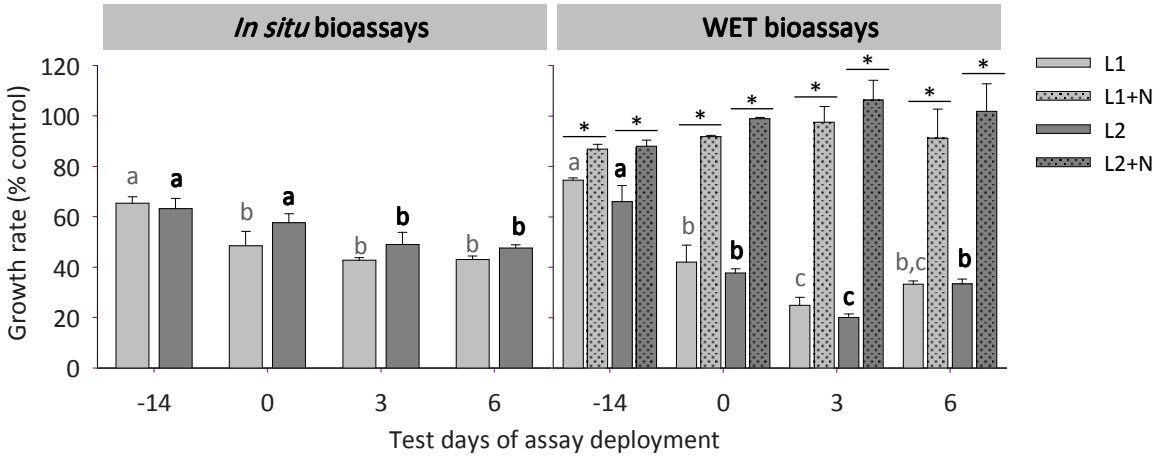


Figure VI.2 - Growth rate of *P. subcapitata* expressed as a percentage of the control, for the *in situ* bioassays deployed at different days on sites L1 and L2. The outcome of WET tests is also presented for site waters without (L1 and L2) and with nutrients (L1+N and L2+N). Error bars represent standard errors. Different letters above error bars indicate values significantly different ($P < 0.05$), when tested within site L1 (light grey letters) and within site L2 (bold letters) along the bioassay deployment days. The asterisks stand for significant differences among the two treatments (without and with nutrients) by a *t*-test, when tested within each site and deployment day.

As pointed out by the two-way ANOVA outcome, there was no statistical differences among the measured growth rates between sites across the testing periods *in situ*, as well as the interaction of the factors testing periods and sites was not significant and, hence, did not explain the variance in the results (table VI.4). In general, a similar response and significance pattern as that for the *in situ* bioassays was achieved in the WET assays, being the testing periods a significant source of data variability (fig. VI.2, table VI.4). Notwithstanding, growth impairments were significantly stronger in the latter bioassays than in the former ones, along the testing periods (table VI.6). The addition of nutrients to both L1 and L2 site water in WET assays significantly enhanced algae growth rates relative to those attained in the corresponding plain site waters, irrespectively of the testing period (fig. VI.2, table VI.6).

High recover percentages (≥ 75.5%) were obtained all through the daphnid *in situ* assays. The feeding rate and survival of both daphnid species determined in each site along the testing periods are presented in figures VI.3 and VI.4, respectively.

Table VI.4 - Summary of the two-way ANOVA applied to algae growth rate (AGR), daphnid feeding rate (DFR) and survival (DS) endpoints exposed to different sites and testing periods.

| Bioassay | Species | Endpoint | Sites | Source of variation | *df* | MS | *F* | *P* |
|---|---|---|---|---|---|---|---|---|
| WET assays | *P. subcapitata* | AGR | - | Testing periods | 3 | 2209.955 | 75.941 | <0.001 |
| | | | | Sites | 1 | 98.255 | 3.376 | 0.086 |
| | | | | Testing periods x sites | 3 | 14.629 | 0.503 | 0.686 |
| *In situ* assays | *P. subcapitata* | AGR | - | Testing periods | 3 | 467.552 | 13.323 | <0.001 |
| | | | | Sites | 1 | 104.114 | 2.967 | 0.103 |
| | | | | Testing periods x sites | 3 | 30.006 | 0.855 | 0.483 |
| | *D. longispina* | DFR | - | Testing periods | 3 | 7378.78 | 11.894 | <0.001 |
| | | | | Sites | 1 | 5039.91 | 8.124 | 0.012 |
| | | | | Testing periods x sites | 3 | 3650.16 | 5.88 | 0.007 |
| | | DS | - | Testing periods | 3 | 5391.15 | 31.537 | <0.001 |
| | | | | Sites | 1 | 5815.33 | 34.018 | <0.001 |
| | | | | Testing periods x sites | 3 | 5061.59 | 29.609 | <0.001 |
| | *D. magna* | DFR | - | Testing periods | 3 | 4278.92 | 3.452 | 0.042 |
| | | | | Sites | 1 | 3024.24 | 2.440 | 0.138 |
| | | | | Testing periods x sites | 3 | 4570.07 | 3.687 | 0.034 |
| | | DS | - | Testing periods | 3 | 640.625 | 11.714 | <0.001 |
| | | | | Sites | 1 | 765.625 | 14.000 | <0.001 |
| | | | | Testing periods x sites | 3 | 807.292 | 14.762 | <0.001 |

Table VI.5 - One –way ANOVA summary for daphnid feeding rate (DFR) and survival (DS) endpoints when subjected to different sites or different periods of test.

| Species | Endpoint | Locals | *df* | $MS_{residual}$ | *F* | *P* | Periods of test (deployment days) | *df* | $MS_{residual}$ | *F* | *P* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *D. longispina* | DFR | L1 | 3,8 | 458.982 | 3.414 | 0.073 | -14 | 1,4 | 3813.614 | 0.118 | 0.748 |
| | | L2 | 3,8 | 781.761 | 12.103 | 0.002 | 0 | 1,4 | 159.495 | 23.848 | 0.008 |
| | | | | | | | 5 | 1,4 | 244.362 | 19.993 | 0.011 |
| | | | | | | | 6 | 1,4 | 2913.724 | 1.773 | 0.254 |
| | DS | L1 | 3,16 | 31.019 | 1.014 | 0.412 | -14 | 1,8 | 8.263 | 1.000 | 0.347 |
| | | L2 | 3,16 | 310.876 | 33.522 | <0.001 | 0 | 1,8 | 105.625 | 198.160 | <0.001 |
| | | | | | | | 3 | 1,8 | 12.343 | 3.679 | 0.091 |
| | | | | | | | 6 | 1,8 | 557.559 | 0.028 | 0.870 |
| *D. magna* | DFR | L1 | 3,8 | 1588.611 | 0.027 | 0.994 | -14 | 1,4 | 1498.130 | 0.010 | 0.925 |
| | | L2 | 3,8 | 890.629 | 9.888 | 0.005 | 0 | 1,4 | 907.461 | 17.624 | 0.014 |
| | | | | | | | 5 | 1,4 | 1008.070 | 0.685 | 0.454 |
| | | | | | | | 6 | 1,4 | 1604.759 | 0.022 | 0.888 |
| | DS | L1 | 3,16 | 31.250 | 1.000 | 0.418 | -14 | 1,8 | 0.000 | 1.000 | 1.000 |
| | | L2 | 3,16 | 78.125 | 18.133 | <0.001 | 0 | 1,8 | 93.750 | 32.667 | <0.001 |
| | | | | | | | 3 | 1,8 | 62.500 | 1.000 | 0.347 |
| | | | | | | | 6 | 1,8 | 62.500 | 1.000 | 0.347 |

Within L1, the feeding rate of daphnids was never significantly affected across the performed assays (table VI.5). On the contrary, within L2, a significant inhibition of daphnid feeding rates took place in

the assays deployed on days 0 and 5 for *D. longispina*, and on day 0 for *D. magna*, being observed a great increase of the overall feeding rates in the following days. As to the one-way ANOVA conducted within each testing period, it was noticeable that the feeding rate of *D. longispina* was significantly different between sites within the testing periods beginning at days 0 and 5, whilst for *D. magna* it occurred just at day 0 (fig. VI.3, table VI.5). According to the outcome of the two-way ANOVA, there was a significant interaction among factors on feeding effects, being the testing periods the major contributor factor for the variance of results relatively to the sites, as greater *MS*- and *F*- values were obtained for the former (table VI.4).

In what concerns the survival of daphnids, it was not significantly affected across the performed assays, within L1. However, within L2, the survival of both daphnid species was significantly affected though only on day 0 (table VI.5). When it turned out to analyse within each testing period, significant differences between sites were detected at day 0 for both daphnid species (fig. VI.4, table VI.5). Again, the two-way ANOVA demonstrated that the interaction testing periods x sites was significant, hence indicating that the differences among sites were dependent on the testing day. Notwithstanding, for the survival results, the factor most responsible for their variance was the sites (table VI.4).

Table VI.6 - Summary of the Student's *t*-test for comparison of algae growth rates within each site and testing day: i) in WET assays conducted with *vs.* without nutrients, ii) in *in situ vs.* WET laboratorial assays.

| Bioassays | Sites | Testing day | *df* | *t* | *P* |
|---|---|---|---|---|---|
| WET assays (with *vs.* without nutrients) | L1 | -14 | 4 | -8.385 | 0.001 |
| | | 0 | 4 | -7.457 | 0.002 |
| | | 3 | 4 | -15.141 | ≤0.001 |
| | | 6 | 4 | -8.644 | ≤0.001 |
| | L2 | -14 | 4 | -5.433 | 0.012 |
| | | 0 | 4 | -35.324 | ≤0.001 |
| | | 3 | 4 | -18.094 | ≤0.001 |
| | | 6 | 4 | -10.463 | ≤0.001 |
| *In situ vs.* WET assays | L1 | -14 | 5 | 2.740 | 0.041 |
| | | 0 | 4 | -0.738 | 0.501 |
| | | 3 | 4 | -5.413 | 0.006 |
| | | 6 | 4 | -0.299 | 0.006 |
| | L2 | -14 | 4 | 0.00951 | 0.993 |
| | | 0 | 5 | -5.122 | 0.004 |
| | | 3 | 4 | -5.754 | 0.005 |
| | | 6 | 4 | -6.430 | 0.003 |

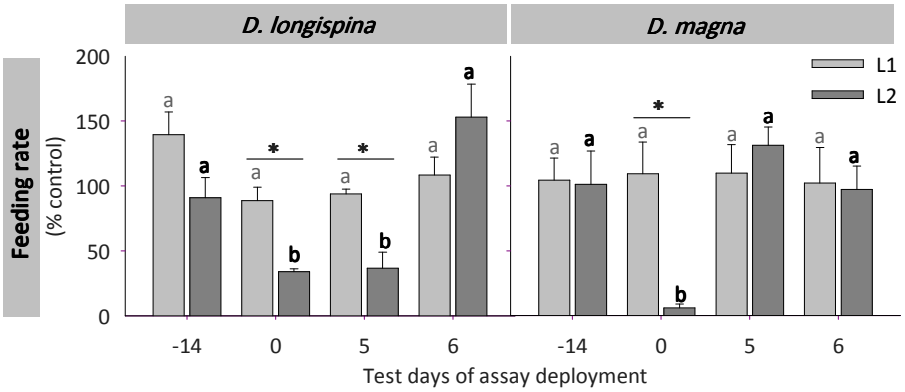


Figure VI.3 - Feeding rate of *D. longispina* and *D. magna* expressed as a percentage of the control, for the *in situ* assays deployed at different days on sites L1 and L2. Error bars represent standard errors. Different letters above error bars indicate values significantly different ($P < 0.05$), when tested within site L1 (light grey letters) and within site L2 (bold letters). The asterisk above two bars represents statistically significant differences between the feeding rates of daphnids on sites L1 and L2, within the same period of testing ($P < 0.05$; *c.f.*, table VI.5).

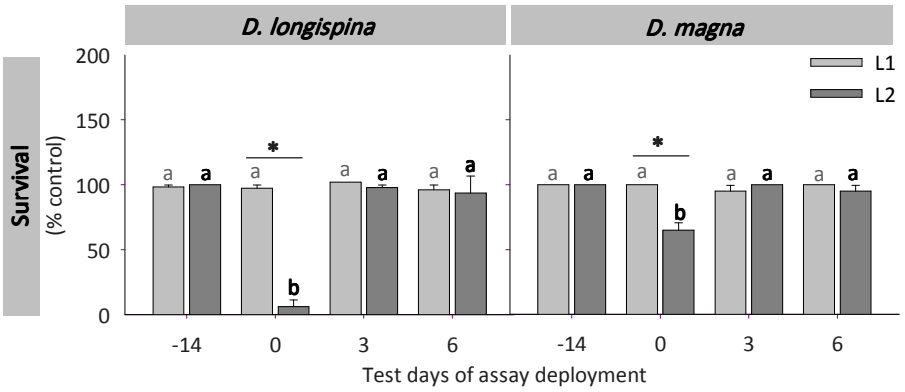


Figure VI.4 - Survival of *D. longispina* and *D. magna* expressed as a percentage of the control, for the *in situ* assays deployed at different days on sites L1 and L2. Error bars represent standard errors. Different letters above error bars indicate values significantly different ($P < 0.05$), when tested within site L1 (light grey letters) and within site L2 (bold letters). The asterisk above two bars represents statistically significant differences between the survival of daphnids on sites L1 and L2, within the same period of testing ($P < 0.05$; *c.f.*, table VI.4).

## 6.4 Discussion

In tandem with the pulsed application of herbicides it was noticeable a general reduction of canal water quality, given the variations recorded for some physico-chemical parameters together with the harmful effects observed on the assessed biological responses (on microalgae and daphnid species).

The values found for pH, conductivity, ammonia, nitrates, nitrites and phosphates were similar to the ones determined in another study also carried out in Lower Mondego Valley (Faria et al. 2007). In general, most of the physico-chemical parameters assumed values that were within the maximum allowable thresholds outlined in national legislation, for surface water quality or irrigation water (MA 1998). However, the dissolved oxygen levels especially in L1, were almost near the established limit (3 mg $L^{-1}$) at day 6, while the individual concentrations of pesticides detected in L2 from day 0 onwards had often surpassed the established threshold (< 0.5 µg $L^{-1}$) (MA 1998).

In spite of the nutrient levels being under the regulated limits, the concentration of nitrogen compounds (*i.e.*, un-ionised ammonia, nitrates and nitrites) and phosphates (table VI.3) for both sites were within the range of total N (TN) and P (TP) defined for mesotrophic (0.70 – 1.5 mg TN $L^{-1}$; 0.025 - 0.075 mg TP $L^{-1}$) and eutrophic (> 1.5 mg TN $L^{-1}$; > 0.075 mg TP $L^{-1}$) states of streams (Dodds et al. 1998). Furthermore, the increase of temperature along the deployed bioassays was accompanied by the aforementioned reduction of [$O_2$], together with the reduction of transparency and a general increase of Chl *a* concentration [[Chl *a*] = 10 - 30 µg $L^{-1}$ as defined by Dodds et al. (1998) for a mesotrophic status] corroborated the mesotrophic condition of both sites, particularly in L1.

Along with the agricultural practices involving the application of agrochemicals on rice fields (*e.g.*, fertilisers and pesticides) nearby L2, not only was observed a surplus of nutrient loads in that site, but also punctual reductions of [Chl *a*] (on days 0 and 5) were noticed. In fact, this latter outcome can be sustained by the herbicide peaks coherently quantified in L2 at the two application moments (test days 0 and 5) (tables VI.2, VI.3), which were likely to reach the aquatic environment through runoff (due to rainfall events between days 0 and 2) [total precipitation of ≈60 mm between test days -7 and 0 (MCTES 2007)], and/or through spray drift (favoured by windy conditions). In the days between major pesticide applications (*i.e.*, days 3 and 6) lower herbicide concentrations were generally recorded, reflecting their rapid degradation. In contrast, considering that L1 was about 2.5 Km upstream from L2, located in the protected wetland where no pesticides were applied, no herbicides were expected to be detected there. Thus, the low concentrations of individual pesticides (< 0.2 µg $L^{-1}$) determined in water samples from L1 could be linked to atmospheric deposition and/or transport processes facilitated by the meteorological conditions above mentioned.

Likewise, it was often noticed that the peak concentrations of herbicides in drainage canals occur right after the treatment of rice cropping fields, being their levels reduced in the following days (*e.g.*, Santos et al. 1998, Santos et al. 2000, Marchesan et al. 2007, Comoretto et al. 2008, Kuster et al. 2008). This was strengthened by the reduced ability to adsorb to soil (low $K_{oc}$) associated to their relatively low degradation half-lives (*c.f.*, table VI.2). Propanil, in spite of its high application rate is rapidly degraded (*c.f.*, table VI.2) after crop treatment, while the concentration of its major persistent metabolite (3,4-DCA) increases in the following days (*e.g.*, Santos et al. 1998). Similarly,

penoxsulam is also quickly degraded under field conditions (Jabusch and Tjeerdema 2008). In turn, quinclorac (Reimche et al. 2008), bentazone and MCPA (*e.g.*, Santos et al. 2000, Comoretto et al. 2008) are reported as being slightly more persistent in drainage ditches nearby rice fields, however, it could be basically attributed to their higher use rates and mobility (low Log $K_{ow}$ and $K_{oc}$) into surface waters relatively to the other herbicides (*c.f.*, table VI.2). The detected concentrations of quinclorac, bentazone, MCPA, propanil and 3,4-DCA in L2 were within the ranges determined in similar aquatic drainage systems: 0.0 – 375 µg $L^{-1}$, 0.02 - 487.5 µg $L^{-1}$, 0.01 - 13.9 µg $L^{-1}$, 1.89 - 71.07 µg $L^{-1}$, and 1.0 - 71.07 µg $L^{-1}$, respectively (*e.g.*, Santos et al. 1998, Santos et al. 2000, Barata et al. 2007, Faria et al. 2007, Marchesan et al. 2007, Comoretto et al. 2008, Kuster et al. 2008, Reimche et al. 2008). To authors' knowledge, however, no study is available concerning the quantification of penoxsulam in surface waters.

Herbicides are generally considered as low toxicity compounds when applied within the recommended rates as they have reduced half-lives, thus presenting a meagre environmental threat (Santos et al. 2000). However, their chemically and biologically active nature linked with their huge and episodic use may counter-act their low environmental persistence, thus leading to toxic effects on non-target aquatic wildlife (Barata et al. 2007), hence reinforcing the need for their *in situ* assessment.

Concerning the microalgae, growth assays gave similar conclusions by comparing to other studies that assessed different *in situ* contamination types (*e.g.*, Twist et al. 1997, Moreira-Santos et al. 2004a) where the use of algae cells enclosed in alginate beads had proven to be a reliable and efficient tool for the contamination assessment of freshwater sites receiving intermittent pulses of agrochemicals. Furthermore, the constructed chambers not only enabled their adequate and successful sub-surface deployment, but also allowed a valid algae growth in control chambers, considering that the validity criteria of microalgae assays were attained.

The significant effects on the growth of *P. subcapitata* when exposed *in situ* in the L1 site (for bioassays deployed on days 0, 3 and 6) was probably related to a decrease of nutrients concentration in spite of L1 advanced trophic state. Nutrient limitation was particularly noticeable for nitrates and nitrites but also the change of TN/TP ratio, since this particular *P. subcapitata* strain has proven to be significantly affected by the reduction of nitrate concentrations and phosphate levels (Gonçalves et al., unpublished). Moreover, the reduced dissolved oxygen levels, the increase in ammonia levels and the stand water conditions recorded along the deployment periods in L1 could have also contributed to the algal growth assays outcomes, once water quality and green algae growth are recurrently known to be affected by these parameters, namely ammonia (*e.g.*, Abeliovich and Azov 1976, Källqvist and Svenson 2003). On the other hand, the dense macrophyte cover of L1 margins (*e.g.*, *Typha latifolia* and *Phragmites australis*) may have influenced algae growth especially due to the

production and release of allelochemicals from microbial degradation of senescing tissues. Allelochemicals are known to occur and accumulate in wetlands due to the typical low topographic variations and reduced water flow, thereby representing harm to green microalgae as was already referred for *C. vulgaris* (Ervin and Wetzel 2003).

The algal growth at L2 showed to be significantly lower for *P. subcapitata* in the assays deployed at days 3 and 6 which was consistent with the reduction of [Chl *a*] and the presence of herbicides and 3,4-DCA in water surface, except for the assay deployed on day 0, where a growth reduction was also observable (fig. VI.2). Vallotton et al. (2008) observed that the effect of a pulse exposure of the herbicide *S*-metolachlor on *Scenedesmus vacuolatus* reproduction depended on the cell development stage. Having in mind that the highest total concentration of the quantified pesticides occurred about 5 days after (≈ 9.4 μg $L^{-1}$) the test deployed on day 0, there is a chance that that pulse did not affect the development phase of *P. subcapitata* cells. Despite the higher pesticide concentrations determined in L2 relatively to L1, those concentrations were still under the ecotoxicological values found for algae growth inhibition (*c.f.*, tables VI.2, VI.3). Under field exposures, however, a series of confounding factors may constrain the interpretation of the bioassay outcome, pre-empting the establishment of cause-effect relationships (Chappie and Burton 1997, Moreira-Santos et al. 2004b). Among the environmental variables potentially limiting algae growth, light, nutrient status and temperature had been pointed out as some of the most critical ones (*e.g.*, Mayer et al. 1998, Moreira-Santos et al. 2004a, 2004b). In order to dismiss their influence WET tests were run under laboratorial conditions with plain and nutrient enriched site water.

WET tests run with plain water from both sites assumed a similar inhibition profile as that occurring for *in situ* bioassays, though it was steepest in the former assays (fig. VI.2). The outcome of *t*-test (table VI.6) had generally reinforced that pattern, indicating that under laboratorial exposures there were significantly lower growth rates relative to the *in situ* responses. Since the *in situ* exposure period was much longer than that of the WET tests and that under field conditions the evaluated herbicides may undergo considerably rapid dissipation (*e.g.*, degradation, adsorption to dissolved suspended solids or organic matter), there is the possibility of algae recovery, hence improving their growth rates along the *in situ* exposure. Yet, a slight increase of nutrient load, particularly in L2 could also enhance algae recovery. Thus, taking into account the coherent inhibitory trend for both assay types, the oscillation of temperature and light intensity along *in situ* assays were not seemingly limiting factors for algae growth.

Notwithstanding, the addition of nutrients to site waters had strongly increased algae growth rates under laboratorial conditions, similarly to what was obtained in other works (*e.g.*, Moreira-Santos et al. 2004b). In L1+N treatment, algae response can reflect some nutrient limitation. However, such explanation does not completely fit for the outcome obtained in the WET assays with

L2+N. Despite the general slight increase of nutrient loads after the preliminary assessment period onwards (table VI.3), the algae growth rates under plain L2 site water had significantly reduced along with the beginning of agricultural practices. Thereby, nutrient limitation did not seem to be the sole factor inhibiting their growth. Indeed, the addition of nutrients in L1+N and L2+N may either diminish the sensitivity of algae to contaminants (Moreira-Santos et al. 2004b) and/or allelochemicals, or trigger a potential masking effect of herbicides. Some studies had noticed that natural water constituents like nutrients and dissolved organic matter may strongly enhance the indirect photodegradation of herbicides (*e.g.,* Halladja et al. 2007, Shankar et al. 2008). In particular, the photolysis reactions undergoing by nitrates and nitrites in water leads to the production of chemical transients, mainly hydroxyl radicals which are extremely reactive oxidants towards herbicide degradation (Halladja et al. 2007, Shankar et al. 2008). Therefore, the surplus of nitrates added to L2 site water in WET assay may have photosensitised the transformation of herbicides therein present, which are already rapidly degraded by photolysis (*c.f.,* table VI.2). Overall, the testing of a treatment with added nutrients must be cautiously interpreted, since it goes beyond environmentally realistic conditions and may lead to an unfeasible understanding of actual *in situ* risks.

The high recovery percentage (≥ 75.5%) of both daphnids species (death or alive) along the testing periods showed that the constructed structures and their *in situ* deployment was successful. That result reinforces the robustness and suitability of *D. longispina* and *D. magna* to be used for the *in situ* bioassessment (Pereira et al. 1999). Accordingly, the evaluated endpoints demonstrated to be sensitive to detect changes in the fitness of daphnids under *in situ* exposures, as it was already verified by different authors (*e.g.*, McWilliam and Baird 2002a, Barata et al. 2007, Damásio et al. 2008).

While in L1 no significant changes were identified for the post-exposure feeding activity of both cladocerans; in L2 a significant inhibition was determined on days 0 and 5 for *D. longispina* and on day 0 for *D. magna* feeding rates. These impairments were in fact consistent with the pulses of herbicides quantified in L2, characterised by the predominance of propanil on day 0 and MCPA, penoxsulam and 3,4-DCA on day 5. Similarly to the results herein achieved, Barata et al. (2007) observed that the levels of pesticide residues measured in drainage canals nearby rice fields like MCPA, bentazone, propanil, molinate and fenitrothion were negatively correlated with the post-exposure feeding rates of *D. magna* deployed *in situ*. Under laboratorial exposures, however, Villarroel et al. (2003) measured a significant filtration depression on *D. magna* exposed to 0.10 – 0.55 mg $L^{-1}$ of propanil, whilst no related studies could be found to the other herbicides and 3,4-DCA. However, dissolved herbicide concentrations in L2 site water were apparently much lower than the ones producing ecotoxicological effects under laboratorial conditions. In spite of this, the occurrence of a rainfall event along with the first application of herbicides on day 0 could have triggered an

increased input of pesticides from adjacent areas via runoff (Schulz 2003, Dabrowski et al. 2005). Accordingly, as already pointed out in other *in situ* studies, the filtration and ingestion of pesticides adsorbed to suspended particles and organic matter may represent a supplementary toxicity route (Tucker and Burton 1999, McWilliam and Baird 2002a, Schulz 2003, Dabrowski et al. 2005). This could particularly hold for penoxsulam and propanil since they presented slightly higher $K_{oc}$ values (*c.f.*, table VI.2) denoting their moderate ability to adsorb.

As discussed by Allen et al. (1995) the impairment of feeding activities may reflect the contamination of feeding apparatus due to the filtration and ingestion of dissolved or particle-sorbed toxicants, or it could be a toxicant-induced energy optimising strategy of daphnids to sustain their survival (DeCoen and Janssen 1998). Invariably, the feeding depression will constrain the energy resources of *Daphnia* sp. and their subsequent allocation for the maintenance of population traits (Allen et al. 1995). As such, the observed depression of D*aphnia* sp. feeding activities, though being roughly restricted to the occurrence of herbicide pulses it may foresee high mortality rates and/or long-term impairments at the population-level.

In fact, whereas no reductions on daphnids' survival were identified in L1, a significant decrease was determined for both daphnid species deployed in L2 at day 0. Such restricted occurrence could be associated to the fact that no survival bioassay was deployed at day 5, hence missing the assessment of potential harmful effects on daphnid survival due to the pesticide peak detected in L2 site water. Nevertheless, the obtained impairment could be an ultimate effect driven by the depression of filtering activities and/or inhibition of ingestion (Allen et al. 1995) as explained above, as long as the environmental concentrations of dissolved chemicals were not enough to directly induce high mortality rates of daphnids (*c.f.*, tables VI.2, VI.3). It could be argued that the runoff occurring along with the rainfall events at day 0 may pre-empt high flow rates and suspended solids, which were regarded as deleterious environmental factors overwhelming feeding and survival of caged invertebrates (Chappie and Burton 1997, McWilliam and Baird 2002a, Dabrowski et al. 2005). However, those conditions were not met in the present study (≤ 0.5 m $s^{-1}$ and 0.76 - 0.94 mg $L^{-1}$, respectively) which were further prevented by the mesh used in chambers. Besides, during the preliminary assay there were also rainfall events that did not significantly reduce daphnids' feeding rates or survival.

Anyway, while the feeding assay provided an earlier and slightly more sensitive response than the survival one (particularly for *D. longispina*), both bioassessment tools provided complementary outcomes. This evidence reinforces their valuable combined use, in order to prevent failing the assessment of contaminants' effects due to endpoint-specificity (McWilliam and Baird 2002b). In what concerns species sensitivity, it was clearly shown that the autochthonous species *D. longispina* was less tolerant to the field impacts than *D. magna*. The lower surface-to-volume ratio of

the standard *D. magna* enables proportionally lower accumulation of contaminants, hence sustaining its higher resistance (Vesela and Vijverberg 2007). Thus, whenever possible, it is recommended the use of autochthonous sensitive species, not only because it enhances the ecological relevance of the study, but also because it provides a feasible and protective understanding of the risks linked to *in situ* contamination near intensive agricultural areas.

In a general view, the detected herbicide concentrations in field water, even in L2, were considerably lower than the ones reported as being deleterious for algae growth and daphnid feeding (for propanil only) and survival (tables VI.2, VI.3). Nevertheless, it should be taken into consideration that either other unmeasured compounds could have accounted for the observed responses, or the presence of low herbicide levels on environmental complex mixtures may elicit synergistic effects, which actually represents an outmost environmental concern according to risk assessors (Cedergreen et al. 2006). Furthermore, the use of formulated herbicides may increase their toxicity to levels much higher than the a.i., due to the presence of adjuvants that increase the efficacy of their mode of action (Pereira et al. in press).

On a whole, the assays with daphnids, especially the feeding rate assay, provided a very sensitive and suitable outcome consistent with the pulses of herbicides in a shorter exposure period compared to that of the algae assays, even if the primary target of that pesticide group is not towards invertebrate species. Nevertheless, the use of both trophic levels enables a more comprehensive overview of intermittent contamination effects on relevant populations responsible for ecosystem integrity. Indeed, during an agricultural season, the impacts generated by pulses of agrochemicals are quite constrained by different factors ranging from the agricultural practices up to the meteorological and environmental conditions. Thereby, the use of *in situ* sub-chronic endpoints involving longer exposure periods provides valuable information, while integrating and accumulating the combined effect of episodic contamination with environmental variables over a time-scale.

## 6.5 Conclusions

In tandem with the beginning and continuing of agricultural practices there was a general impairment on the aquatic system quality, mainly triggered by the input of herbicides and nutrients.

Overall, the species and endpoints used were suitable and sensitive for the *in situ* assessment of temporary impacts on aquatic drainage canal generated by the intermittent application of agrochemicals on rice fields. As such, the inhibition of the biological responses (*i.e.*, algae growth, daphnids' feeding and survival) determined for the caged organisms deployed in the most impacted site (L2) was generally consistent with the concentration pulses of herbicides, though an increased toxicity source for daphnids was attributed to the potential filtration of particle-bound contaminants

due to runoff events. In spite of this, following herbicide peaks, the whole system was seemingly able to quickly recover towards less deleterious conditions, given the smoothing or lack of effects on organisms' responses. The outcome of algae WET assays with plain site water provided significantly stronger inhibitory responses relatively to those obtained *in situ*, for both sites. The addition of nutrients resulted in significant growth increase. Notwithstanding, such results should be carried out with cautious, due to potential masking effects.

The use of sensitive autochthonous species and different levels offered an ecologically relevant picture of the potential risks that natural populations may undergo. Moreover, daphnids' feeding rate was the most sensitive endpoint giving an early-warning indication of harmful aquatic conditions. Nevertheless, future works should also address the study of potential community structure fluctuations to boost the knowledge of water quality variations.

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# Chapter VII

Final remarks

# Final remarks

Although great efforts have been done towards the broaden implementation of Integrated Production programs in Europe, namely associated with the use of less toxic pesticides (EEA 2005, Boller et al. 2004) there is still an evident concern about the environmental threats posed by intensive agricultural practices. Indeed, different environmental compartments (*e.g.*, soil, water) adjacent or within the agricultural areas undergo continuous direct and/or indirect exposure of pesticides, thus constraining the health of non-target individuals, what in turn may compromise the agro-ecosystem integrity. Furthermore, an additional concern arises when agricultural areas are located in the vicinity of ecosystems presenting high ecological value that renders them a protected-area designation in the Natura 2000 Network, at the light of EU directives (EEC 1979, EEC 1992). Actually, this is often the scenario found nearby or within farming areas (*e.g.*, rice field areas) in Europe, and more particularly in Portugal, which justifies a proper assessment of pesticide impacts on biodiversity loss and ecosystem functioning (Tarazona and Sánchez 2006, Macedo-Sousa 2009). At the European level, such awareness led to the establishment and/or proposal of directives demanding the protection of aquatic (EC 2000) and soil (CEC 2006) resources, being the use of pesticides regulated by legislation (e.g., EEC 1991, EC 2006) that require the implementation of risk assessment procedures described in support guidance documents (EC 2002a, EC 2002b, EC 2003). However, in Portugal, the assessment plans are roughly limited to the physical, chemical and sometimes biological scrutiny of exposed environmental compartments, being frequently neglected the study of potential ecotoxicological effects on non-target organisms (Macedo-Sousa 2009), with subsequent effects on populations and communities.

The present study is a contribution to improve the understanding of ecotoxicological effects driven by the use of pesticides, mostly herbicides, on terrestrial and especially freshwater non-target organisms. As a way to increase the environmental relevance of this work, some tasks targeted sub-areas (A1 and A2) of an extensive Portuguese agricultural area (Lower Mondego river Valley), which sustains a high biodiversity, being one of the sub-areas (A2) located near a protected wetland. Complementary assessment tools were thereby applied towards a tiered and refined evaluation of pesticide effects, being generally retrieved an indication of changes in the ecosystem quality during the agricultural season.

The first experimental approach (chapter II) was carried out in a rice cropping farm, Quinta do Seminário (sub-area A1), aiming the preliminary screening of the quality of natural samples from two sites (VE and RP), during the drainage of rice fields. Besides the organic enrichment observed in water samples, no pesticides were found in water and elutriates from both sites. Yet, the toxicity screening of natural samples with WET assays evidenced that water from the canal crossing rice

fields (VE) was more deleterious for microalgae growth, particularly for *P. subcapitata* than that from the river (RP). In turn, sediment elutriates were less toxic for microalgae growth, being a possible cause attributed to the absence of toxic contaminants and their high nutrient contents. Once it was found that this sub-area A1 was constrained by other diffuse contamination sources from upstream husbandry places, it was dismissed from the subsequent work.

The tasks that followed this preliminary study were then directed to the sub-area (A2) within the Lower Mondego river Valley, in which the dominant source of diffuse pollution was addressed by agricultural practices, like the overuse of agrochemicals. As such, it could provide more straightforward conditions to reach the proposed goals, while seemingly reducing the complexity and uncertainty of environmental data interpretations. The sub-area A2 is essentially used for rice production, though corn is also cropped but in less extent. Having this in mind, the herbicides selected for further ecotoxicological analysis were Viper (a.i. penoxsulam) and Mikado (a.i. sulcotrione), which were being respectively applied on local rice and corn cultures. In a way to generate unavailable baseline ecotoxicological information that could help estimating potential hazards induced by environmental exposures to those formulated herbicides, laboratorial tests were conducted with individual and mixture compounds measuring biological responses of freshwater microalgae and daphnids (chapter III). Viper was one to three orders of magnitude more toxic than Mikado for all species tested, as well as for acute (immobilisation of daphnids) and chronic (*i.e.*, algae growth, daphnids' growth and reproduction) endpoints. The advanced explanation for that toxicity was based on the presence of toxic adjuvants, which may enhance the negative effects of the a.i. on non-target biological receptors (Tominack 2000, Cox and Surgan 2006). On the other hand, CA and IA model predictions indicated significant deviations from additivity for the mixture toxicity of those formulated herbicides. Considering that CA is the model pertaining a conservative estimation under a regulatory standpoint (Cedergreen et al. 2008, Syberg et al. 2008) it was foreseen the occurrence of antagonistic effects for *P. subcapitata* and *C. vulgaris* growth, and *D. longispina* survival when subjected to realistic environmental levels of mixture of pesticide residues. For *D. magna*, however, synergistic effects were expected to occur in their immobilisation if exposed to environmental mixture concentrations of Viper/Mikado, considering the tested mixture ratio. Notwithstanding, mixture effect studies are still in its infancy, being thereby generally accepted the need for further pharmacological studies in tandem with ecotoxicological studies covering more comprehensive mixture designs and different sub-lethal endpoints in order to decrease misunderstanding conclusions (Syberg et al. 2008).

The toxicity screening of the individual a.i.s (penoxsulam and sulcotrione) and formulated (Viper and Mikado, respectively) herbicides on the avoidance behaviour of terrestrial earthworms (chapter IV) showed the same trend aforementioned for freshwater organisms, in that Viper was also

the most toxic product followed by its a.i. penoxsulam in Lufa 2.2, in spite of its target of action being absent in animals (Roberts et al. 2003). In order to boost the ecological relevance of this particular task, natural soils from the rice and corn fields were used as substrates to test the toxicity of the formulated products. Nevertheless, only the rice field soil was applied for Viper toxicity testing due to the reduced habitat function quality of the corn field soil. The final outcome strengthened that the organic matter and clay/silt contents of the rice field soil could have reduced the bioavailability of chemicals (Ying and Williams 2000, Römbke et al. 2005, Garcia et al. 2008), hence slightly reducing the gauged toxicity for Viper. Overall, the tested concentrations were yet beyond the application rates, thereby suggesting that the risk of those herbicides for *E. andrei* may be low if those rates are respected.

Stepping into an approach with higher environmental relevance, the evaluation of natural samples from in-crop (*i.e.*, soil elutriates from the paddy field) and off-crop (*i.e.,* water and sediment elutriates from the adjacent canal) environments of the sub-area A2 revealed, as expected, the overall organic enrichment of samples, and the detection of pesticides residues[3] in sediment elutriates, mainly during the culture season. This degradation of paddy/water system quality was associated to the agricultural practices and/or the proximity of sites to the farming areas (chapter V). The WET assays showed the strong inhibition of microalgae growth, particularly during the cropping season, being elutriates the most deleterious natural samples relatively to the water samples. This outcome was corroborated by the physico-chemical survey, though the pesticide levels in L2 and L3 elutriates, during that season, were below the ecotoxicological values (Tomlin 2000, Junghans et al. 2003, Cedergreen and Streibig 2005) and established benchmarks (MA 1998). Therefore, two possible explanations for algae growth inhibition were mentioned: i) the synergistic effect of chemical mixtures occurring in L2 and L3 elutriates, and ii) the high un-ionised ammonia contents in L1 and L2 elutriates, which are recurrently mentioned as being toxic beyond certain levels (*e.g.*, Källqvist and Svenson 2003, Koukal et al. 2004). The decrease in algae growth recorded for water samples, particularly during the rice culture in L1-W were hypothesised to be a result of allelopathic effects, linked to the dense macrophyte cover of that site. In opposition, the life-history traits of both daphnid species obtained in WET assays with water and elutriates were generally significantly stimulated during and before the cropping season, similarly to other published works (*e.g.*, Viganò 2000, Antunes et al. 2007a, Antunes et al. 2007b). Yet, it was observed an apparent rebound of the ecosystem before the culture season (chapter V), in what concerns the physical and chemical parameters, and algae responses.

[3] It should be mentioned that like in A1 (chapter II), halogenated and polycyclical aromatic hydrocarbons were also identified in A2 (chapter V) probably due to agricultural engines, but the detected values were inferior to respective benchmarks (whenever available; *c.f.*, table in annex), and thus, they were not considered within the discussion of chapter V.

Going through higher tier assessments, the development of *in situ* assays has been recommended under ecological risk assessment frameworks (*e.g.*, Boxall et al. 2002), as well as they have been considered as a reliable tool for estimating diffuse agricultural contamination (*e.g.*, Tucker and Burton 1999, Phillips et al. 2004). Likewise, the *in situ* bioassays carried out in the sub-area A2 retrieved a reasonable picture of potential impacts on non-target freshwater populations of algae (*P. subcapitata*) and daphnids (*D. longispina* and *D. magna*), mainly driven by the input of nutrients and pulses of herbicides a.i.s such as penoxsulam, bentazone, propanil and MCPA, along with the intensification of agricultural practices nearby L2 (chapter VI). The feeding inhibition of daphnids, especially that of the autochthonous species *D. longispina* provided a rapid sign of increased toxicity in L2 probably due to the filtration of particle-bound pesticides; whereas the effects on the survival of both species were restricted to the first day of pesticide application, in tandem with rainfall events. In L1, however, the responses of both daphnid species were unaffected. In turn, the reduction of *P. subcapitata* growth in L2 reflected the accumulation and integration of peaks of herbicides and nutrient changes occurring over a time-scale, while in L1 the inhibitory responses were potentially linked to some nutrient and oxygen limitation or allelopathy. Despite the detected concentration of herbicides in L2 surpassed the allowed thresholds for national surface water protection (MA 1998), they were below the ecotoxicological values determined under laboratorial conditions (*e.g.*, for Viper; chapter IV). Notwithstanding, low levels of pesticides in complex environmental mixtures, as well as the use of formulations presenting different dispersible abilities and adjuvant types may work together to induce synergistic effects (chapters III and IV).

Two common traits regarding the impact of agricultural activities in Lower Mondego river Valley could be highlighted from the studies carried out in sub-areas A1 (chapters II) and A2 (chapters V and VI): the enhanced trophic state occurring in the adjacent aquatic systems, as well as the general detection of pesticides with relatively quick degradation rates. The diffuse input of fertilisers is one of the main factors contributing for the organic enrichment of aquatic systems that may end up in their eutrophication. This is actually a major challenge for the conservation of freshwater resources and protection of biodiversity status (Carpenter et al. 1998, Smith et al. 1999, EC 2000, EEA 2005), especially concerning protected wetlands, which lentic conditions tend to accelerate the eutrophication process, as it was observed for L1 site (chapters V and VI).

On the other hand, taking into account that the agriculture in Lower Mondego river Valley is following an Integrated Production strategy, it was expected that the applied pesticides would present a low risk for the environment. This is because the compounds are generally characterised for their lower persistence, adsorption and bioaccumulation abilities, being usually rapidly degraded. However, the contamination of surrounding environmental compartments, namely the aquatic system was mainly ruled out and limited to the agricultural season, being the most negative effects

concomitant to the intermittent inputs of high herbicides' loads. Indeed, this was reinforced by the studies presented in chapters II, V and VI, in which a few days/weeks after the application of pesticides the ecosystem could seemingly recover, given the lowering of their concentrations and/or the reduction of harmful effects on the responses of non-target organisms. Anyway, knowing that the system of canals/ditches supplying the agricultural areas of Lower Mondego Valley are ultimately drained into Mondego channel and river, it should be taken into account that the diffuse pollution from those areas may also be risky for human activities (*e.g.*, recreational, fishing, irrigation purposes) that depend on those receiving freshwater systems, particularly during the cropping season.

On a whole, through the evaluated tiers it was possible to confirm the existence of hazardous conditions associated with the farming practices in Lower Mondego Valley, especially for sensitive sub-lethal biological responses (*e.g.*, feeding inhibition, growth and reproduction) of autochthonous species (*e.g.*, *P. subcapitata* and *D. longispina*), which had invariably increased the reliability and relevance of predictions of ecological changes. However, additional analyses, biological inventories, and ecotoxicological endpoints will be required to improve the feasible determination of the effective risks for the ecosystem and to allow a more solid definition of cause-effect relationships. These relationships are actually quite difficult to establish with specificity whenever the assessment studies are focused on environmental exposures, along with a series of confounding factors that are likely to underestimate or overestimate the sole effect of pesticides under assessment. Yet, the background challenge of such evaluations is that all variables are integrated towards a final outcome that definitely gives a closer overview of real impacts.

One of the striders enclosed in this work is associated with the generation of acute and chronic ecotoxicological data for formulated herbicides, which are normally overlooked in available literature, though they are the ones entering into different environmental compartments during their application. Furthermore, this study is a contribution for a site-specific risk assessment, being the used tools already required under regulatory documents towards the monitoring and protection of natural resources (*e.g.,* EC 2000). As such, the resulting information could be integrated into a future retrospective ERA directed to the target study sites, as well as it could help in the (re)definition of assessment plans directed to other Portuguese areas subjected to extensive agriculture. Anyway, the whole study may offer baseline data for local and regional management entities towards the creation of environmentally-supported mitigation programs, in order to achieve the European leading goals of biodiversity and habitat protection, jointly with the conservation of existent natural resources.

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# Annex

Table presenting the xenobiotics (ng $L^{-1}$) determined in sediment elutriates from sites L1, L2 and L3 during the rice culture (study described in chapter V). The available benchmark values for each chemical are presented on the shadowed columns.

| Chemical compounds | Chemical group | Elutriates with ASTM | | | Elutriates with MBL | | | Benchmarks for surface waters | |
|---|---|---|---|---|---|---|---|---|---|
| | | L 1 | L 2 | L 3 | L 1 | L 2 | L 3 | EPA (1) | National (2) |
| Phenantrene | PAH | < 0.20 | 2.35 | 2.36 | < 0.20 | 3.21 | 4.63 | 3.0E+04 | |
| Anthracene | PAH | < 0.20 | <0.20 | 6.25 | < 0.20 | <0.20 | <0.20 | 3.0E+02 | |
| Chrysene | PAH | < 0.20 | 5.18 | 5.81 | < 0.20 | 6.47 | 7.01 | 7.0E+03 | 1.0E+05 |
| Benzo(b)fluoranthene | PAH | < 0.20 | 7.12 | 4.96 | < 0.20 | 5.13 | 5.93 | - | |
| Benzo(k)fluoranthene | PAH | < 0.20 | 2.12 | 2.58 | < 0.20 | 2.74 | 4.64 | - | |
| Benzo(a)pyrene | PAH | < 0.20 | 1.63 | 2.12 | < 0.20 | 2.09 | 3.12 | 14 | |
| Hexachlorophene | PCB | < 0.20 | 4.78 | 4.83 | < 0.20 | 3.01 | 4.83 | - | - |

(1) http://rais.ornl.gov/tools/eco_search.php

(2) MA (Ministério do Ambiente). 1998. Law by Decree no. 236/98 de 1 de Agosto. Estabelece normas, critérios e objectivos de qualidade com a finalidade de proteger o meio aquático e melhorar a qualidade das águas em função dos seus principais usos. Ministério do Ambiente, do ordenamento do Território e do Desenvolvimento Regional, Diário da República – I Série-A, Nº 176: 3676-3722.